BIOLOGICAL CONTROL OF THE NEMATODE PARASITES OF SHEEP AND CATTLE BY A NEMATODE-TRAPPING FUNGUS

by

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ABSTRACT

Chapters one to three review literature relevant to the thesis. The first chapter deals with the nature of nematode parasitic disease in livestock, the factors affecting its distribution and frequency of occurrence in Australia, and the production losses caused by these parasites. The second chapter deals with the effects of parasitism on the host animal including changes to metabolism and physiology caused by parasitic disease. The epidemiology of nematode-parasitic disease is examined and control methods discussed, particularly with regard to the past reliance on anthelmintics and the emergence of anthelmintic-resistant parasitic nematodes. Alternatives to the use of anthelmintics, including breeding for parasite resistance in livestock, vaccination and biological control, are discussed. Chapter three contains a review of the literature pertaining to nematophagous fungi and their potential as biological control agents against animal-parasitic nematodes. Chapter four introduces the experimental program conducted during the project and the following five chapters contain procedures used and the results of these experiments. Results are discussed in chapter ten. A survey of soil samples taken from Tasmanian sheep-grazing properties examined the frequency of occurrence of nematode-trapping fungi in sheep pastures, and the fungi isolated were compared for their ability to grow and trap nematodes on laboratory media. The predaceous fungus, *Arthrobotrys oligospora,* was chosen for use in further experiments. The effects of fungal inoculum density and prey population size on fungal predacity were examined in chapter six. Chapter seven reports on investigations into fungal spore formulations including attempts to encapsulate fungus for protection within the ruminant stomach and methods for collecting fungal spores from mature grain cultures. Chapter eight reports on *in vitro* and *in vivo* experiments on the ability of fungal spores to survive passage through the digestive tract of sheep and cattle. Chapter nine reports on glasshouse trials in which various fungal treatments were applied to faeces from a parasitised sheep on pasture swards. Yields of infective larvae on pasture herbage were compared. Soil inoculation had little effect on pasture infectivity but when faeces were sprayed with a suspension of spores, reductions of up to 90% were obtained. The timing of the spray in relation to deposition of faeces had a significant effect on the ability of the fungus to reduce the numbers of infective larvae reaching the herbage.

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Introduction

Several years ago I kept dairy goats for a hobby and made a modest income from milk sales. During that time the debilitating effects of parasitic worms became evident to me, as did the need for repeated anthelmintic treatment of animals which rapidly acquired new infections from grazing contaminated pastures. Some time later, while working at the CSIRO with Dr Robin Bedding on a project concerning entomopathogenic nematodes, I encountered the nematode-trapping fungus, *Arthrobotrys oligospora,* which occasionally interfered with my experiments by destroying large quantities of infective juvenile nematodes. It was then that I became interested in the possibility that the free-living stages of animal-parasitic nematodes could be susceptible to such a predatory fungus.

A literature search revealed that not only are these pre-parasitic larvae susceptible, but this has been the subject of research in France, Denmark, and now Australia, over the last ten years. Livestock producers have relied upon the use of anthelmintics for the last thirty years, since the introduction of effective broad spectrum products. The overuse of these chemicals has resulted in the emergence of resistant parasite populations and there is now an urgent need to integrate the use of anthelmintics with other forms of parasite control including pasture management, breeding for resistance, and possibly biological control.

The approach taken in this project was to find a way of introducing a predatory fungus to depress the numbers of infective parasite larvae on pasture herbage. In the early stages of the project a small field trial was set up at Bothwell, where pasture was dusted with crushed grain cultures of predatory fungus and subsequent worm burdens in new-born lambs were monitored by faecal egg counts over the following weeks. Results were inconclusive as few lambs developed infections. Later in the project, glasshouse trials revealed that the introduction of fungus to the soil has little effect on pasture infectivity.

Previous workers have shown that predatory fungi can reduce the numbers of infective larvae arising from faeces by 90% or more, but most of this work has been done in faecal cultures or using artificial cowpats. This project examined the effects of applying the fungus in a spray directly to whole faeces from an infected sheep. Significant reductions in subsequent pasture infectivity were obtained by this method.

CHAPTER 1

THE NEMATODE PARASITES OF SHEEP AND CATTLE

Introduction

The nematode parasites of sheep and cattle are one of the most important animal health problems in Australia. They cause not only death and misery in livestock during disease outbreaks, but also more insidious losses to meat and wool production from sub-clinical infestation. They have been the subject of much veterinary research giving rise to several monographs, from which the following discussion is drawn. Strictly speaking, they are in the province of the veterinarian or parasitologist, but it is the practicing agriculturalist who has to manage their control, using whatever methods are available to him. The following discussion is intended to provide understanding relevant to the agriculturalist, while perhaps not a complete review of the topic from a veterinary or parasitological viewpoint.

1.1. Animal-parasitic Nematodes

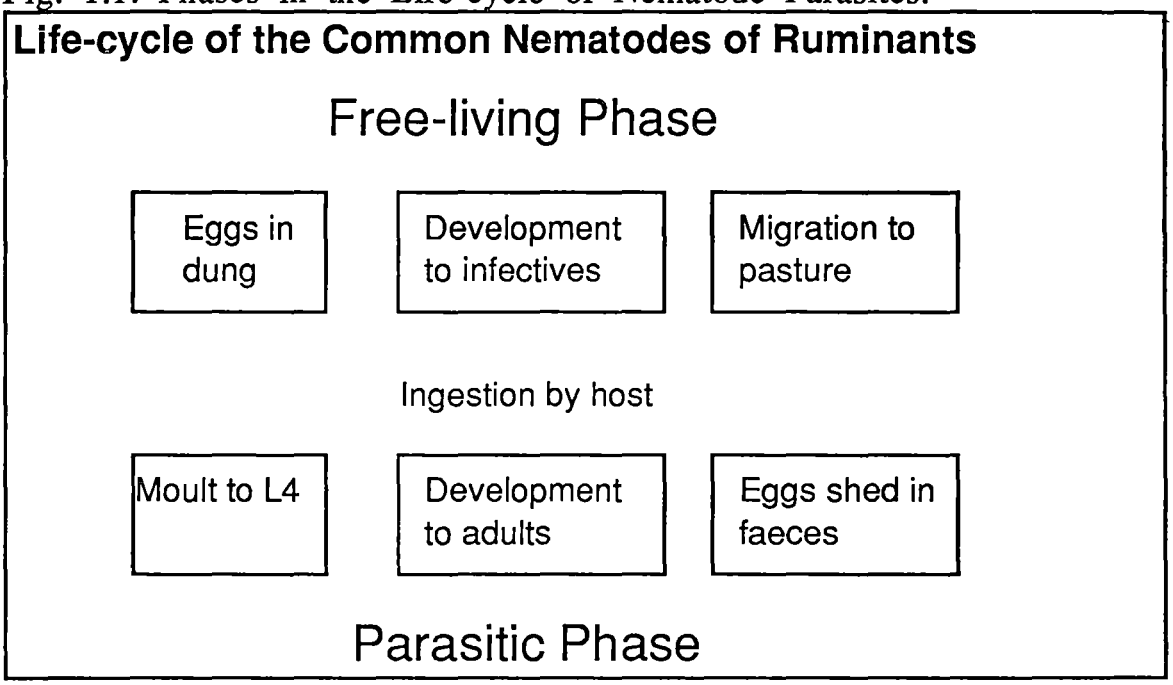
Animal-parasitic nematodes are commonly known as roundworms and may affect many parts of the body (Riley, 1949), though most of the species of concern to animal production infect the gastro-intestinal tract and lungs. The major pathogenic gastrointestinal trichostrongyles of domestic ruminants belong to the genera *Ostertagia, Haemonchus* and *Trichostrongylus* , for species living in the abomasum, while the important species inhabiting the small intestine belong to the genera *Cooperia, Nematodirus and Trichostrongylus.* (Cole 1986).

Their life-cycle is direct (there is no intermediate host) and includes a free-living, as well as a parasitic, phase. The free-living phase culminates in infective juvenile larvae, which contaminate pasture herbage. It is the factors affecting survival of the infective stage larvae that determine the frequency of occurrence of nematode species and the severity of infection they produce in livestock. Trichostrongylosis causes economic losses in animal production worldwide (Larsen, 1991).

Life-cycle

Typically the nematodes have six stages in their life-cycle; the egg, four larval stages and the adult (Croll & Matthews 1977). The gastrointestinal parasites of ruminants have a biphasic life-cycle; the first three larval stages are free-living and are the means of transmission between hosts.

Fig. 1.1. Phases in the Life-cycle of Nematode Parasites.



Eggs hatch releasing first stage larvae (L1) which feed and grow until they moult to the second stage (L2) larvae. L1 and L2 larvae are microbivorous, and remain in the faeces until they reach the third larval stage, which is the infective stage. The second stage cuticle is retained as a sheath and the infective larva has no openings for mouth or anus. The duration of development from the egg to infective stage is 5 to 7 days under optimum conditions, but may be about 18 to 20 days under field conditions. Appearance of infective larvae on pastures may be delayed as migration depends on adequate moisture. In field experiments conducted in Western Victoria, Callinan (1978a, 1978b, 1979) found the mean development time to maximum yields of L3 on herbage varied between 30 and 40 days for larvae of *Ostertagia circumcincta, Trichostrongylus axei* and *T. vitrinus*. The first appearance of L3 on herbage was from as little

as 4 days after egg deposition, but mean minimum development times were 12 to 14 days.

The life-cycle is completed when the infective larva is ingested by a susceptible host and moults to enter its fourth larval stage. The L4 travels to the abomasum or intestine where it enters the mucosa and feeds until it reaches adult stage, when it emerges into the lumen and proceeds to feed, mate and reproduce. Sometimes, as in late spring *Ostertagia* infections, the L4 becomes inhibited in the mucosa and does not complete its development until the following autumn, causing type II ostertagiosis. Otherwise, the time from infection to the appearance of eggs in the host's faeces is about 20 days. In the northern hemisphere, type I ostertagiosis is normally seen in grazing calves during the summer and early autumn of their first grazing season. The type II disease normally occurs during late winter or early spring and is caused by the synchronous maturation of large numbers of larvae, inhibited at the early L4 stage. In the southern hemisphere type II disease appears in the same months as in the northern hemisphere, in this case late summer and early autumn (Waller, *pers. com.*). Inhibition of L4s was considered to be mediated by host immunity until Armour and Bruce demonstrated the phenomenon in parasite-naive calves, inoculated with *Ostertagia* larvae which had been subjected to chilling at 4°C for 8 weeks (Armour and Bruce,1974). This indicated that the inhibition was due to larval factors induced by chilling rather than host immunity.

1.2. Factors Affecting Occurrence

Climatic factors are the most important determinants of disease occurrence, and the distribution of parasites over the continent reflects temperature and rainfall patterns. Cole (1986) provided a full description of regional distribution in Australia and listed the species of economic importance for each area. Most species occur in all areas, but certain species are more common, or occur more frequently, depending on the type of climate. This is illustrated by the list of common species occurring in summer- and winter-rainfall areas in table 1.2.1. (adapted from Cole, 1986).

Cole described how climate affects the livestock industry in four major ways, which have an indirect affect on parasitic disease. "It affects the types of vegetation and pastures grown in different areas, their seasonal growth, the geographical distribution of livestock and their grazing density, and the productivity of livestock." The development of nematode eggs and larvae takes place in dung on the ground, and it is the factors affecting this that determine the seasonality of larval availability.

Conditions Favouring Development

Generally, development to infective stage is confined to those months that have a mean temperature above 10°C, though for optimum development, temperatures of 15°C or higher are required. Adequate moisture is the other requirement, and in summer, dryness will frequently kill the majority of eggs and early larval stages before they can reach the more resistant infective stage. Freezing temperatures will halt larval development and may be fatal to some species of parasite, and in winter, temperatures may be too low for development. Conditions for optimum development are most likely to in spring and autumn, with rainfall determining the extent of summer development and mild temperatures moderating mortality during winter.

It is possible for the free-living parasites to accumulate at different stages of development during adverse conditions. For example, *Trichostrongylus* spp. can endure extended periods of dry conditions in the embryonated egg stage, hatching when adequate moisture becomes available, while the infective larvae of most species can survive in faeces for considerable time and will not migrate to pasture until there is adequate moisture. Cole (1986) quotes estimates of 50mm rainfall in a month in New England and 75mm a month in Queensland as adequate for hatching and development.

Table 1.2.1.

Commonly Occurring Parasites of Sheep and Cattle.

Sheep Parasites	*Summer-rainfall*	*Winter-rainfall*
Haemonchus contortus	+++	+
Ostertagia spp.	++	+++
Trichostrongylus spp.	+++	+++
Nematodirus spp.	++	++
Oesophagostomum columbianum	++	-
Oes. venulosum	+	+++
Chabertia ovina	+	+++
Dictyocaulus filaria	++	++
Fasciola hepatica	+++	+++
Cattle Parasites		
Haemonchus placei	+++	+
Ostertagia spp.	+	+++
Trichostrongylus axei	+	+++
Cooperia pectinata	+++	+
C. punctata	+++	+
C. oncophora	+	+++
Bunostomum phlebotomum	+++	+
Oesophagostomum radiatum	+++	-
Dictyocaulus viviparus	+	++

+++ High incidence
++ Medium incidence
+ Low incidence

1.3. Movement of Infective Larvae

The infective larvae of many parasitic nematodes require a thin water film for movement, relying on the force of surface tension to provide the purchase necessary for propulsion. Deeper water films reduce the nematodes movements to ineffectual thrashing. Croll (1970) discussed the physics of nematode locomotion and the effects of water films of varying thickness on speed of travel. He gave examples also of nematodes which are able to swim freely and others that can move independently of free water, although the latter usually emerge in damp weather. The ability of infective larvae of animal-parasitic forms to swim is readily observed in the laboratory, but this form of movement is probably of little significance to their dispersal in migrating out of the dung.

1.4. Inactivity and Quiescence

In the absence of stimuli or the onset of unfavourable conditions, such as dehydration or cold, nematodes cease activity, and may even cease metabolic activity. This adaptation conserves energy and extends the lifetime of infective larvae. Quiescence may be defined as a temporary, environmentally induced state of inactivity which ends when the environment again becomes favourable or includes a suitable host (*ibid.*). This adaptation enables Strongyloide infective larvae to survive many weeks longer than would otherwise be expected in the pasture environment.

1.5. Distribution over Pastures

The distribution of numbers of infective larvae in random samples of pasture is highly overdispersed (Donald, 1967), because the faeces from which larvae arise tend to be clumped, and the larvae are known to migrate only short distances laterally (*ibid.*).

1.6. Survival of Free-living Larvae

1.6.1. Abiotic Factors

The major determinants of survival are temperature and moisture. Adaptations for survival vary between species, as the following examples illustrate.

During the early larval stages, temperature has a greater effect on survival of *Trichostrongylus colubriformis* larvae than does moisture, given that the soil is moist enough for development to take place (Gibson and Everett, 1967). Temperatures below 10°C occurring after development has begun kill most larvae before they hatch. The larvae of *Ostertagia circumcincta* are able to develop at low temperatures (Gibson and Everett, 1972) and tend to remain in the faeces for long periods, maintaining populations on the herbage at a steady level, while *T. colubriformis* larvae leave the faeces within 2-4 weeks of deposition of eggs. Young (1983) found that *O. circumcincta* larvae migrated in abundance after much smaller falls of rain than were required for migration

by *O. ostertagi* larvae. A study of *Nematodirus* spp. in Greenland, (Rose, 1990) found that low temperatures did not prevent infective larvae appearing on the herbage within one month in summer. Eggs did not develop synchronously because development beyond the morula stage could be delayed for up to 2 years.

Dry conditions occurring after egg deposition can kill developing larvae, but the infective L3 stage of both *T. colubriformis* and *O. circumcincta* are able to survive on herbage for 20 weeks or longer (Gibson and Everett 1972). Callinan (1978a, 1978b, 1979) found no survival over summer of the L3 larvae of *O. circumcincta, T. axei* or *T. vitrinus,* but at other times of the year the L3 larvae of *T. vitrinus* persisted in faeces up to 106 days and on herbage and soil up to 208 days.

1.6.2. Biotic Factors

Donald (1994) reviewed the effects of biological agents on nematode parasites, which, unlike other pests, have no documented cases in which natural enemies from another geographic region have been introduced to gain control. The activities of introduced dung beetles have been shown to reduce peaks of infective larval availability on pasture by up to 94%. However, the beetle populations are subject to seasonal effects, and there have been no controlled experiments on the effects dung beetles have on the seasonal pattern and intensity of nematode infections or on animal production.

Nematophagous fungi offer potential for exploitation as control agents, and considerable research has been conducted towards that end (reviewed by Grønvold, 1989; Hashmi & Connan, 1989; Waller & Larsen, 1993). This will be more fully examined in chapter 3.

1.7. Conclusion

Gastrointestinal parasitism of ruminants is a problem occurring in all production regions where rainfall is sufficient for parasite development. Adults are parasitic, but the juveniles are free-

living and constitute the means of transmission between hosts. The predominant species vary between winter- and summer-rainfall areas according to their adaptations to cold and dryness. The severity of disease and production losses are related to the level of intake of infective larvae. The availability of infective larvae on pasture is controlled by seasonal conditions, and there are few reports of natural enemies having significant impact on nematode survival. Introduced dung beetles can reduce pasture contamination dramatically but they are subject to seasonal influences. Nematophagous fungi may have potential as biocontrol agents.

CHAPTER 2

EFFECTS OF PARASITISM AND CONTROL METHODS

2.1. Introduction

The physiology of parasitic disease has been studied extensively in sheep, because sheep are economically important to the wool industry. Much of this work has been carried out at the Moredun Reasearch Institute, Scotland, and the CSIRO McMaster Laboratory, Sydney, and forms the basis for Symons' monograph (Symons, 1989). These investigations have been based on induced infections of the abomasal parasites, *Ostertagia circumcincta* and *Haemonchus contortus*, and the parasites of the intestine and colon, *Trichostrongylus colubriformis, Nematodirus battus* and *Chabertia ovina* .

During the course of infection the gastrointestinal tract becomes physically damaged by the parasites (Symons 1989). Anorexia, malabsorption and losses of endogenous proteins and minerals are induced by the infection, and grazing behaviour may be affected (Fell *et al.* 1991). One significant feature of the infection is leakage of plasma into the gastrointestinal tract, and this can account for up to 10% of the total plasma volume per day (Holmes and Coop, 1994).

2.2. Physical Effects of Parasites

Development of *O. circumcincta* larvae takes place within the abomasal epithelium (Symons 1989). Nodules form around infected crypts as irritation causes oedema and inflammation. After 10 days (in lambs), larvae leave the swollen glands and develop to adults on the mucosal surface. The larvae of *H. contortus* create gastric pits as they move to and from the mucosa of infected lambs. Epithelial cells become damaged and lose their contents. Integrity of tight junctions between cells is impaired allowing leakage of plasma into the lumen and pepsinogens into the plasma. Blood may be lost through ulceration or sucking by the parasites.

Intestinal parasites such as *T. colubriformis* damage the absorptive epithelium of the intestine. The fourth stage larvae mature in the crypts of Lieberkuhn, or burrow among the villi of the small intestine.

Changes in abomasal pH can permit bacteria to survive passage into the small intestine, causing enteritis and diarrhoea to further complicate malabsorption. The physical damage to abomasal epithelium from *O. circumcincta* in lambs, results in oedema and ulceration, accompanied by a rise in pH to as much as 7 or 8, from loss of parietal cells. Digestion of proteins may be impaired by increased abomasal pH.

2.3. Effects on Nutrition

Research workers at the Moredun Research Institute measured utilisation of nitrogen, minerals and metabolisable energy, and others at the McMaster Laboratory measured nitrogen utilisation in infected lambs (Symons 1989). Nitrogen balances were reduced, particularly in the first two to three weeks following infection with daily doses of 4,000 *O. circumcincta* larvae. After a single dose of 30,000 *T. colubriformis* larvae, wethers had reductions in nitrogen balance of 30% in days 21-30, and 17% in days 31-40. Metabolisable energy retention was reduced by infection also. Interestingly, drenching with an anthelmintic on days 21, 42, 63 and 84, did not improve the utilisation of metabolisable energy in lambs subjected to daily infection with 5,000 *O. circumcincta* larvae.

The hosts mineral balance becomes affected (Symons 1989). Anaemia may develop as a result of blood loss from either haemorrhage or blood sucking by adult parasites. This places increasing demands on erythropoiesis and can cause iron deficiency. Both abomasal and intestinal infections impair skeletal growth in lambs (Symons 1989). Trichostrongylosis induces low plasma phosphorus levels due to loss of endogenous phosphorus and impaired absorption. Calcium uptake is not impaired, but there is increased faecal loss. The effects of this are osteoporosis and poor skeletal growth. Mineralisation of tooth

enamel can be affected by severe infections. Sodium deficiency, indicated by salivary Na:K ratios of less than 5, may be caused by mixed infections at abomasal and intestinal sites (Holmes and Coop, 1994), with hypomagnesaemia resulting from the altered saliva composition raising rumen K concentrations sufficiently to impair Mg absorption. Suttle and Brebner (1990) found parasitised lambs voluntarily increased their intake of a mineral supplement to compensate for a nematode-induced sodium deficit.

2.4. Anorexia

The majority of gastrointestinal nematodes cause a reduction in voluntary feed intake, the degree of inappetance being dependent on the level of infection (Holmes and Coop, 1994). The mechanisms are considered to be multifactorial, and recently correlations have been made between changes in serum gastrin and cholecystokinin levels, gut motility and reduced appetite. Symons (1989) mentions a number of pair-feeding experiments designed to determine the importance of anorexia in causing production losses in parasitised sheep. Results varied between nematode species and the production parameter measured. For example, *T. colubriformis* infection reduced wool growth by 40% compared to pair-fed sheep, and caused poor skeletal growth in lambs. On the other hand, in lambs infected with *O. circumcincta*, anorexia accounted for poor skeletal growth.

2.5. Nitrogen Metabolism

Damaged tissue and leaked plasma-proteins contribute to loss of endogenous nitrogen into the intestinal lumen (Brown *et al.* 1991). Even though 87% of this nitrogen may be reabsorbed (Symons, 1989), diversion of nitrogen metabolism away from wool and muscle growth for the repair and maintenance of gastrointestinal epithelium and plasma-proteins, compromises the efficiency of nitrogen utilisation in parasitised animals. These effects are proportional to the severity of infestation; animals receiving low weekly doses of infective larvae show no significant loss of function (*ibid.*).

Brown, Poppi and Sykes, (1991), used post-rumenal protein infusions (via abomasal catheter) to determine the limiting factors on the efficiency of feed utilisation of lambs infected with *Trichostrongylus colubriformis* . Nitrogen retention was increased fourfold by the protein infusion, and this treatment also reduced the mean faecal egg output and mean total parasite count. Replacing the protein infusion with an equivalent energy supplement only partly increased the rate of energy retention. The results indicated the effects of worm infestation were caused through endogenous protein losses in the gastrointestinal tract.

2.6. Effects of Parasitism on Production

The effects of chronic parasitism on sheep were demonstrated by Southcott *et al*, (1966), who compared efficiency of wool production, measured in grams of wool per kg organic matter digested, between Merino ewes either suppressively drenched or not treated. They found dry matter intake increased 19.2% in parasitised ewes while wool production fell by 7.3%, depressing the gross efficiency of wool production by 23.4%.

Sheep at 12 per acre had higher faecal egg counts and greater food intake than sheep at lower stocking rates. Unfortunately these sheep had less available feed per acre also, so it is unclear whether the increased worm burden resulted from the higher stocking rate or lower feed availability. The lower availability of feed per acre, although it did not restrict intake, must have meant the sheep were eating shorter grass, probably increasing their intake of infective larvae. The greater dry matter intake of the animals at the higher stocking rates could also have been due to grazing behaviour, with larger flocks spending more time grazing than groups of less than five sheep (Southcott *et al*, 1962), but the authors did not report flock sizes used.

Others have found parasitism to depress feed intake; for example, Sykes, Poppi and Elliot (1988) found both intake and energy retention were depressed by infections of *Trichostrongylus colubriformis* and *Ostertagia circumcincta*. These sheep were individually penned indoors, and therefore free of any grazing behaviour effect. However, isolation itself may have influenced

results from these sheep; it has the ability (van Adrichem & Vogt, 1993) to alter heat production and energy balance relationships. Direct comparison between pen trials and grazing trials may be complicated by this. There is agreement, however, that parasites depress the efficiency of production, whether this is measured in liveweight gain or wool growth.

Parasitism may also affect sheep behaviour; Fell *et al* (1991) found that a mild infection with *Haemonchus contortus* changed the behaviour of mature Merino ewes, infected sheep showing increased locomotor activity when penned but not when at pasture, although overall the penned sheep had twice the activity of the pastured sheep. Although intake fell and liveweight dropped in penned sheep during the initial ten days from commencement, these effects could not be attributed to infection. The challenge infections resulted in low parasitism levels and no evidence of disease.

Anorexia was responsible for reduced weight gain in calves subjected to nematode parasitism in a field grazing experiment using a sustained release bolus to protect control animals (Bell *et al.* 1988). Since this trial did not use housed animals, it clearly implicates parasitism as the cause of the depressed feed intake.

Wool production of wether weaners given moderate daily doses of *T. colubriformis* may be reduced to 40% that of worm-free, pair fed controls (reports cited by Cole, 1986), and liveweight gains in suppressively drenched lambs can be 37% to 48% greater than in their parasitised paddock mates (*ibid.*). Although fewer trials had been conducted using cattle, Cole (1986) was able to present data comparing the growth of Hereford weaners over 12 months in which gains in the treated and untreated groups averaged 233kg and 134kg, respectively, as well as two other trials in which drenching produced increases in weight gains of lesser amounts.

2.7. Effect of Level of Intake of Infective Larvae

Rumball (1991) used a creep system to give lambs limited access to clean pastures, and compared faecal egg counts and liveweight

gains between groups of lambs at three different levels of larval intake. Faecal egg counts in lambs on pastures cleaned with a summer hay crop followed by cattle grazing, were 8% of controls, and their liveweight gains were 121% of controls. While the creep lambs had lower egg counts (3280 epg compared to controls 4215 epg), and greater liveweight gain, this could have been due to the nutritional value of the creep forage rather than the lower worm burden.

Other studies have shown the effects of nematode parasitism on productivity in sheep and cattle vary with the level of larval intake (Symons,1989). In lambs dosed weekly with varying numbers of infective larvae, reduced weight gain occurred during the first four weeks only in those lambs receiving the highest dose of larvae. After 16 weeks of continual infection, the rates of gain were similar in all groups. Lambs infected with *Ostertagia circumcincta* received four times the number of larvae given to the lambs infected with *Trichostrongylus colubriformis* . Despite this, the effect on bodyweight was greater with *T. colubriformis*, indicating the greater pathogenicity of that species. Concurrent infections had more than additive effects. This is important because under natural conditions mixed infections are more common than monospecific infections.

Reproduction and lactation may also be depressed by nematode infections. Ewes treated with anthelmintics three weeks before mating produced 4.4% more total lambs and 3.2% more live lambs than untreated ewes under field conditions in New Zealand (Symons 1989). A loss of milk production has for some time been known to be associated with parasitism in milking cows, goats and ewes. The worm burdens of adult cows are usually too low to have any effect of production, but intake of infective larvae causes losses of plasma proteins at the site of the immune reaction in the gut epithelium (Armour, 1994). Treatment of dairy cows with anthelmintics does not significantly increase milk production (Cole, 1986). In this situation, control of pasture infectivity would be needed to prevent production losses.

It is clear that low weekly doses of infective larvae have very little effect on sheep and could be beneficial, in so far as they stimulate the animals' immune system. The tolerable intake rate varies between species, according to pathogenicity.

2.8. Host Resistance

The development of resistance or immunity to infection is an important outcome of a parasitic challenge. The factors affecting this include intrinsic factors of age, lactation, breed and differences within breed (Cole 1986). Extrinsic factors are the species of parasite, the size of the stimulating dose of infective larvae and the nutritional status of the host.

Lambs under six months old are less capable of developing strong protective immunity than older lambs, but the age at which this ability develops varies between individuals (Donald and Waller, 1982). Resistance diminishes in reproductive ewes during late pregnancy and lactation, to a degree which is subject to genetic variation. Together, these animals constitute a pool of susceptible individuals in the population every year. Similar patterns have been noticed in cattle (Cole, 1986). The evidence for genetic variation comes from three sources: variation among breeds, variation within breeds and the identification of genes contributing to the variation (Stear and Murray, 1994).

Parasite species vary in their ability to stimulate immunity (Cole 1986). Weaner sheep normally develop strong resistance to *Trichostrongylus* after a period of infection, and this nematode seldom seriously affects adult sheep.Immunity to *Haemonchus* infection, however, seems to be less permanent, and this parasite may also affect older animals under conditions favouring infection.

The size of the larval dose required to stimulate immunity can generally be described as moderate (Cole 1986), but that dose must be continued for a minimum time (100 days in the case of *T. colubriformis*). Provided that the infection is not too severe, and nutrition is adequate, immunity begins to develop after about 8 weeks (Symons 1989). This will cause a reduction in

faecal egg count, but adult parasites will be retained unless an anthelmintic is used. Plasma leakage due to enteric damage is highest in week 12, by which time immunity is fully developed. Normal function has returned by week 24, except in the case of the most heavily parasitised animals. Nutrition is important both for the establishment and maintenance of immunity, and its interaction with parasitism is complex.

2.9. Interaction of Nutrition and Resistance

Protein status affects the development of resistance to *Haemonchus* (Cole 1986), *Ostertagia* and *Nematodirus* (Holmes and Coop, 1994) and alleviates the effects of infection with *Trichostrongylus colubriformis* (Brown *et al.* 1991). Gulland and Fox (1992) found that experimentally induced infections in well-fed, housed, Soay sheep had little effect, while their wild flock-mates were suffering high mortality as a result of similar infections combined with malnourishment. A dietary molybdenum supplement reduced the number and size of adult *T. vitrinus* in lambs exposed to infection, and caused a lowering of proteinase activity in these worms (Suttle *et al.* 1992). Molybdenum increased or enhanced the inflammatory response in the intestinal mucosa to *H. contortus* infection in lambs, and reduced adult parasite numbers and faecal egg counts in treated individuals (*ibid.*).

Symons (1989) reported the moderating effect of nutrition on parasitised lambs. A high-protein diet prevented weight loss in *Haemonchus contortus* -infected lambs during the first six weeks of infection.

2.10. Epidemiology

The factors affecting outbreaks of severe parasitic disease are complex and interacting. As discussed above, climate has a major effect on parasite occurrence, and seasonal conditions affect survival, development and migration to pasture of infective larvae. The numbers of infective larvae per kilogram of grass can be diluted by rapid pasture growth or magnified by larvae being concentrated onto short grass at times of poor pasture growth.

Infective larvae tend to remain close to dung pats, and livestock tend to avoid feeding from these places unless they have no option. Resistance to infection is reduced in malnourished animals, and in wild populations, parasitic nematodes may act as a regulator when numbers begin to outstrip food supply (Gulland and Fox, 1992).

Gordon (1948) suggested that the following factors contributed to epidemics:
1. The introduction of an organism to a susceptible community.
2. The susceptibility of an already infected community being increased.
3. The introduction of susceptible animals into an already infected community.
4. An increase in the infective dose of the parasite in an already infected community.

Examples of the causes of these epidemics on a grazing property would be:
1. The introduction of infected animals onto a property previously free of the parasite.
2. Stress such as general malnutrition reducing the immune response in a flock.
3. The arrival of new-born lambs or the movement of previously unchallenged stock to infected pastures.
4. Environmental conditions favouring development, survival or migration of infective larvae on pasture.

The first three circumstances can be anticipated and dealt with by proper management. It is the last which is the most common, and least predictable, of the causes of parasitic epidemics. The numbers of infective juvenile worms present on the vegetation affects the level of intake of infective larvae by grazing animals and thus also affects the severity of parasitism. Pasture infectivity is the term given to the number of larvae per kg of dry matter. The dry matter intake of grazing animals varies with physiological status as does their susceptibility to nematode parasites. The most susceptible animals are the young,

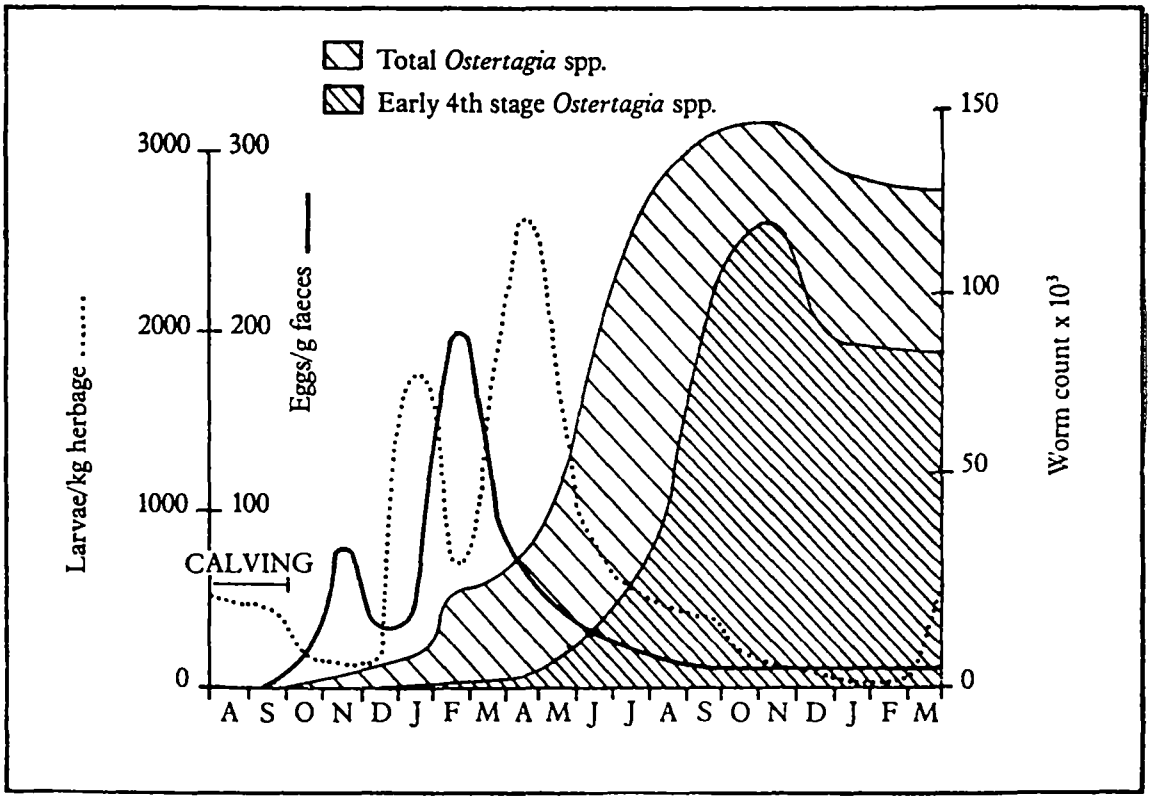
particularly during weaning, when their dry matter intake must increase to meet their nutritional needs.

The factors operating in the system are:
1. The numbers of infective larvae per unit area of pasture.
2. The amount of herbage over which the infectives are distributed.
3. The intake of the animals grazing the herbage.
4. The susceptibility of the animals grazing the herbage.

Cole (1974) reported the factors affecting the availability of infective larvae on pasture as:
(a) seasonal occurrence of rainfall
(b) species of parasite present
(c) level of egg deposition on pastures
(d) time of year when most egg deposition occurs
(e) occurrence of extremes of heat, cold and dryness.

The epidemiology for any region will tend to follow a seasonal pattern, but this varies between regions and is affected by weather patterns within any particular season. However, monitoring feacal egg counts and pasture infectivity over a

Fig 2.9.1. The association between calving time, faecal egg counts, larval availability and the build-up of *Ostertagia* worm burdens in calves (from Brunsdon 1980).



number of seasons has been useful in understanding the dynamics of disease and developing control strategies within regions (Smeal, 1982). When pastures are short and nutrition is marginal, parasite intake is likely to increase as infective larvae are concentrated onto available grass. Animals grazing longer grass may ingest very few larvae` as these tend to be on shorter vegetation below the top cover (Gordon 1948). The rate of intake is affected by selective grazing (Cole 1986), but invariably, a rise in the numbers of larvae on a pasture grazed by susceptible animals is accompanied by a rise in the numbers of eggs in their faeces. The figure 2.9.1. produced by Brunsdon (1980) illustrates the relationship between pasture contamination, larvae per kg herbage and faecal egg counts of *Ostertagia* in calves. Peaks of faecal egg counts are followed by peaks of larvae on herbage about four weeks later. The summer peak of pasture infectivity is followed by peak faecal egg counts three weeks later, but host immunity prevents the autumn peak from having a similar effect. Adult worm numbers reach their peak in autumn, and although parasite numbers continue to rise until the following spring, most recruits are inhibited at the L4 stage.

"Outbreak weather" is associated with exceptionally heavy rainfall following a dry period (Cole 1986). This is due to release of infective larvae and eggs which have accumulated within dung over the dry spell.

2.11. Pasture Contamination

Gordon (1948) was one of the first to emphasise the importance of pasture contamination in the disease cycle. He estimated that "a sheep heavily infested with *Haemonchus contortus* may harbour 3,000 females, each laying 5,000-10,000 eggs per day, giving a daily output of 15 - 30 million eggs. A sheep heavily infested with *Trichostrongylus* spp. may harbour 20,000 females, each laying 200 eggs per day, giving a daily output of but 4 million eggs. A sheep heavily infested with *Oesophagostomum columbianum* may harbour 200 females, each laying 12,000 eggs per day, giving a daily output of 2.4 million eggs." Obviously, if only a small proportion of these eggs develop to infective larvae, a pasture can become heavily infested over a short time. These

estimates are exaggerated, as recent studies have shown that the numbers of adult females establishing in a host from a single dose of infectives are affected by the size of the infecting dose (Fleming, 1988). Fleming found female *Haemonchus contortus* in heavily parasitised sheep were less fecund than those in less heavily infected animals. When the worm burden contained 722 female adults, these each produced 3,500 eggs per day, but in sheep containing 2,900 females fecundity fell to 855 eggs per female per day. Gordon's estimated output of 15-30 million eggs per day is an order of magnitude too high, but even at 2.5 million eggs per day, pastures would become rapidly contaminated.

Various attempts have been made to produce models of nematodiasis (reviewed by Smith and Grenfell, 1994). Callinan and Morley (1982) used daily weather data to predict the nematode status of weaner sheep in western Victoria with the "Nemat" model. They used the model in computer experiments to evaluate different drenching strategies. A model developed for nematodiasis in lambs in New Zealand (Leathwick *et al.*, 1992), was used to determine the relative importance of factors in determining parasite dynamics. They found that climate, affecting survival to L3 and migration of L3s to pasture, was more important than the survival rate of L3s on herbage, in parasite dynamics. Large differences in faecal egg output induced by changing the rate of development of host resistance to infection resulted in only minor differences in pasture larval populations. Varying worm fecundity however, resulted in a related response in pasture populations. The general pattern of parasite dynamics within the host was largely explained by the hosts ability to resist infection, whereas the dynamics of pasture populations were determined largely by climatic factors.

2.12. Control Methods

2.12.1. Anthelmintics

Prior to 1962, most anthelmintics had a narrow spectum of activity and were generally of low efficiency. The compounds used were arsenic, copper sulphate, copper sulphate and sodium arsenite, copper sulphate and nicotine sulphate, carbon

tetrachloride, tetrachlorethylene, piperazine, trichlorphon (still in use), coumaphos, bephenium compounds and methyridine (Cole 1986). The first highly efficient broad-spectrum anthelmintic was thiabendazole, introduced to Australia in 1962. A number of others soon followed, including the benzimidazoles - parbendazole, cambendazole, oxibendazole, mebendazole, fenbendazole, albendazole, oxfendazole; pro-benzimidazoles - thiophanate, febantel; other broad-spectrum anthelmintics - tetramisole, levamisole, pyrantel, morantel; narrow-spectrum anthelmintics - naphthalophos, rafoxanide and closantel.

Anthelmintic treatment programs for sheep have been designed to suit specific rainfall zones (Cole 1986). For winter-rainfall areas treatments in early- and mid-summer may be followed by a third for weaners in autumn, before the return of moister conditions. In the uniform rainfall zone recommendations are similar, and in the summer-rainfall areas treatments in autumn (weaning, pre-mating), late winter - early spring (pre-lambing) and late spring - early summer (post-lambing) are recommended.

The situation for cattle is less clear-cut. In Queensland, parasitic gastroenteritis is cause by *Haemonchus placei, Oesophagostomum radiatum, Cooperia punctata, C. pectinata* and *Bunostomum phlebotum*. Ostertagiasis is seldom a serious problem, although it does occur in some areas. Regular monthly treatment with anthelmintics is the only effective way of dealing with the disease when the infection rate is high, as occurs on many dairy farms, and in places where grazing management to reduce infection is not practiced. In the extensive grazing areas, where most beef cattle are located, the situation is quite different. Low stocking rates keep infection rates low, and little benefit is obtained from treatment except in circumstances producing exceptional infectivity, as may occur after soaking rain (Cole 1986).

Ostertagiosis is a problem for both beef and dairy cattle in more southerly locations.

The high level of efficiency and relative safety of anthelmintics led to their intensive use in intensive livestock operations (Waller 1992). This caused a rapid selection for resistance (Waller 1986), which is now ubiquitous in all livestock industries (Waller 1992). Integrated parasite control schemes, which combine some sort of grazing management with anthelmintic treatment, have had poor adoption rates by farmers (*ibid.*).

While anthelmintics are losing their effectivity due to resistance, their properly controlled use still offers protection to grazing sheep. Local eradication of *Haemonchus* is possible (Barger *et al.* 1991) using closantel, and, combined with a quarantine period for incoming stock, can remove the need for further drenching. Although resistant worms are widely distributed (Love *et al.* 1992; Waller 1992), mixtures of anthelmintics can still be effective (Anderson *et al.* 1991). Ivermectin has proven effective against worms resistant to benzimidazole and levamisole, but resistance to this drug has already been detected (Le Jambre 1993). Programs such as Wormkill (developed for the Northern Tablelands of NSW) can reduce the use of anthelmintics (Barger *et al.* 1991) and thus prolong their useful life. Ivermectin is a macrocyclic lactone, chemically distinct from another macrocyclic lactone, moxidectin, which has greater potency against the three genera of nematodes usually involved in resistance (*Haemonchus*, *Ostertagia* and *Trichostrongylus*). Use of Moxidectin should be carefully controlled (Kieran 1994) to prevent development of resistance to this drug. Kieran suggests goats as a likely source of resistant worms and recommends their exclusion from treatment with Moxidectin.

2.12.2. Problems for Long-term Control

Anthelmintics have been chronically overused in the past, resulting in development of resistance in worm populations (Prichard, 1990, 1994; Waller, 1992). Their use also impacts on non-target species such as fungi and invertebrates, which are important in the degradation of dung. While the effect of ivermectin residues in dung is insufficient to reduce blowfly populations (Mahon and Wardhaugh, 1991), there may be environmental consequences for pastureland due to delayed

breakdown of dungpats and destruction of dung beetles (Wall and Strong, 1987). Controlled release capsules (CRCs) of benzimidazoles effectively destroy incoming parasites but not adults (Barger, Steel and Rodden, 1992). Their use, particularly as reported in goats (McDougall 1992), could increase the rate of resistance development in worms.

Alternate grazing can reduce pasture infectivity to negligible levels but relies on the parasites being benign in the alternate host. Reports of *Trichostrongylus axei* causing deaths and weight loss in sheep (Abbott and McFarland, 1991), and *Ostertagia ostertagi* causing clinical disease in naturally infected sheep (O'Callaghan *et al.* 1992), are warning signs that increased use of alternate grazing could have adverse consequences.

2.13. Anthelmintic Resistance

Anderson and Waller (1985) reviewed the methods for detection of resistance in parasitic nematodes, biochemical and genetic mechanisms of this resistance, methodologies for assessing the impact on animal production systems of anthelmintic-resistant parasites, and suggestions for integrated control measures to delay the development of resistant parasite populations. In another review, Pritchard (1994) cited reports of resistance to thiabendazole dating back to 1964. Similar reports have come from Australasia, Africa, Europe, and both North and South America, and there are now reports of resistance to anthelmintic drugs wherever animals have been regularly treated and investigations made (*ibid.*).

Benzimidazole resistance has been reported in *Haemonchus contortus, Nematodirus spathinger, Nematodirus filicolis, Nematodirus abnormalis, Teladorsagia (Ostertagia circumcincta/Teladorsagia trifurcata/Teladorsagia davtiani), Trichostrongylus axei, Trichostrongylus colubriformis* and *Trichostrongylus vitrinus* in sheep in South Australia and in *Trichostrongylus axei* and *Cooperia oncophora* in cattle from Australia and New Zealand.

Levamisole and morantel have similar modes of action and resistance to one is often associated with resistance to the other (Prichard, 1990). Overt resistance to levamisole indicates a higher level of resistance to both drugs than resistance to morantel only. Levamisole resistance has been reported in *Haemonchus contortus, Trichostrongylus colubriformis* and *Ostertagia circumcincta* in sheep and *Ostertagia ostertagi* in cattle in Australia (Prichard, 1994).

Ivermectin resistance has appeared in *Haemonchus contortus* in sheep and goats in South Africa, Brazil and the USA, and appears to be developing in *Haemonchus contortus,* and*Trichostrongylus colubriformis* in Australia (Le Jambre, 1993; Prichard, 1994).

Because of widespread resistance to broad spectrum anthelmintics in *Haemonchus contortus*, closantel is used to control this parasite in sheep and goats (Prichard, 1994) with local eradication being possible (Barger *et al.*, 1991). Unfortunately, resistance to closantel also has been reported in South Africa and Australia (Prichard, 1994).

2.14. Breeding for Parasite Resistance in Livestock

The use of the animals' immunological resistance to parasites, is important in any control program, and the CSIRO's Animal Health division has been selecting since the mid 1970s for resistance to *Haemonchus* in one sheep flock and *Trichostrongylus* in another (Taylor 1993). The University of New England has been selecting its "Golden Ram" flock since the early 1980s. In normal flocks, the most resistant 50% of sheep produce less than 10% of faecal eggs, while the most susceptible 15% produce 50% of the eggs contaminating pastures. Researchers believe that sheep from the three resistant flocks would require no drenching under commercial conditions, and have begun trials to test this hypothesis (Taylor 1993).

In the same article, Taylor reported evidence suggesting genetically inherited resistance to one species of parasite confers useful levels of resistance to others. It has been argued that the short generation time of nematodes will allow them to rapidly

adapt to increased resistance in sheep, thus negating years of breeding effort. Taylor reports, however, that after 14 generations of selection by passage through resistant sheep, there was no difference in the ability to infect resistant sheep or normal sheep. Taylor (1993), estimated that inclusion of parasite resistance in a selection index could increase the rate of genetic gain in productivity by 10% annually.

2.15. Vaccines

The stimulation of the immune system by vaccination would be an ideal way of controlling worms. Lambs injected with preparations of adult *Haemonchus* developed resistance resulting in greatly reduced egg outputs and total worm burden (Munn *et al.* 1993), but the response to protein extracts obtained by different techniques varied greatly. Taylor (1993) reported that three donor sheep were required to provide sufficient adult worm material to vaccinate one animal, an obvious difficulty in commercialising such a vaccine. Larvae attenuated by radiation, induced up to 98% protection against challenge infection by *Dictyocaulus viviparus* in cattle (Lightowlers, 1994). The success of this vaccine in Europe has been repeated in India, where *Dictyocaulus filaria* is a significant pathogen of sheep. In a review of this topic, Lightowlers (1994) suggested that recombinant antigen vaccines are likely to provide protection against several parasites in the near future, although achievements in this field have lagged behind vaccines against other organisms. He also proposed that the expression of vaccine antigens in recombinant plants may offer protection to grazing animals in the future.

CHAPTER 3

NEMATOPHAGOUS FUNGI

3.1. Introduction

The nematophagous fungi are a group of microfungi with adaptations enabling them to feed on nematodes. They include species which grow inside the victim's body and may be regarded as pathogens or endoparasites, and others which are free-living and capture their victims in specialised hyphal traps of various kinds. On the basis of their functional characteristics, Barron (1977) classified these fungi into two ecological groups:

a) Predacious fungi - produce specialised trapping hyphae on their mycelium.

b) Endoparasitic (endozoic) fungi - invade and grow inside nematodes.

A third group was proposed by Nordbring-Hertz (1988):

c) Egg parasitic fungi - which attack the egg stage, particularly of cyst and root-knot nematodes of plants.

The hyphae of the endoparasitic fungi are contained within the bodies of their hosts, except for reproductive structures such as evacuation tubes and conidiophores which extend into the outside environment. Some of these fungi produce motile zoospores which actively track down their victims using a chemical gradient and could thus be considered predators, but generally, the distinction can be made on the basis that the endoparasites exist outside nematodes' bodies only as spores (Barron 1977).

3.2. History

The first description of a nematophagous fungus was by Fresenius (1852), of a fungus growing on organic debris. The

ability of this fungus, *Arthrobotrys oligospora,* to produce specialised hyphae, was discovered by Woronin (1870), but the function of these net-like bails was not understood until Zopf (1888) found that they trapped nematodes. Almost fifty years later Dreschler (1933, 1933a) showed that a powerful adhesive was responsible for the capture of active nematodes in the nets. Barron (1977) provided a fascinating review of how developments in our knowledge of predacious fungi have followed improvements in techniques for observing microscopic organisms. The advent of transparent agar media permitted Dreschler to make his observations. Elucidation of the events following capture and penetration of nematodes by fungal hyphae continue to progress with improvements in microscopy (Veenhuis *et al.*, 1989; Murray and Wharton, 1990).

3.3. Taxonomy

Most of the approximately 200 species of nematophagous fungi belong to the class Deuteromycetes, the imperfect fungi, with the exception of a few belonging to the classes Zygomycetes and Basidiomycetes. Their taxonomy has been covered in several comprehensive reviews and articles (Soprunov, 1958; Cooke and Godfrey, 1964; Haard, 1968; Jarowaja, 1970; Barron, 1977; Schenk, Kendrick and Pramer, 1977; Peliolle, 1979; Domsch, Gams and Anderson, 1980; Van Oorschot 1985; Gams 1988). Cooke and Godfrey (1964) provided a useful key to their identification.

Arthrobotrys Corda is a genus of Hyphomycetes, containing 12 species (Haard, 1968), some of which are distributed world-wide. The distinguishing characteristic was described by Corda (1839) as the formation of conidia on sterigmata in a whorled pattern at the tip and nodes of the conidiophores. Conidiophores were described as simple, erect and septate, and the conidia as two-celled, with an apiculiform base.

3.4. Morphology

Conidia of the predatory fungi are much larger than those of the endoparasites because they must contain sufficient energy to grow a thallus or at least a trap before feeding can commence,

whereas the spores of endoparasites are either ingested or adhere to the cuticle of their victims, whose bodies are invaded immediately after germination. Adaptations to a nematophagous existence have resulted in specialised spores among the endoparasites and hyphal adaptations which comprise the traps of the predators. These adaptations are familiar to nematologists and have been described and illustrated by Barron (1977), see fig. 3.4. The following brief descriptions are derived from that text as well as personal observations.

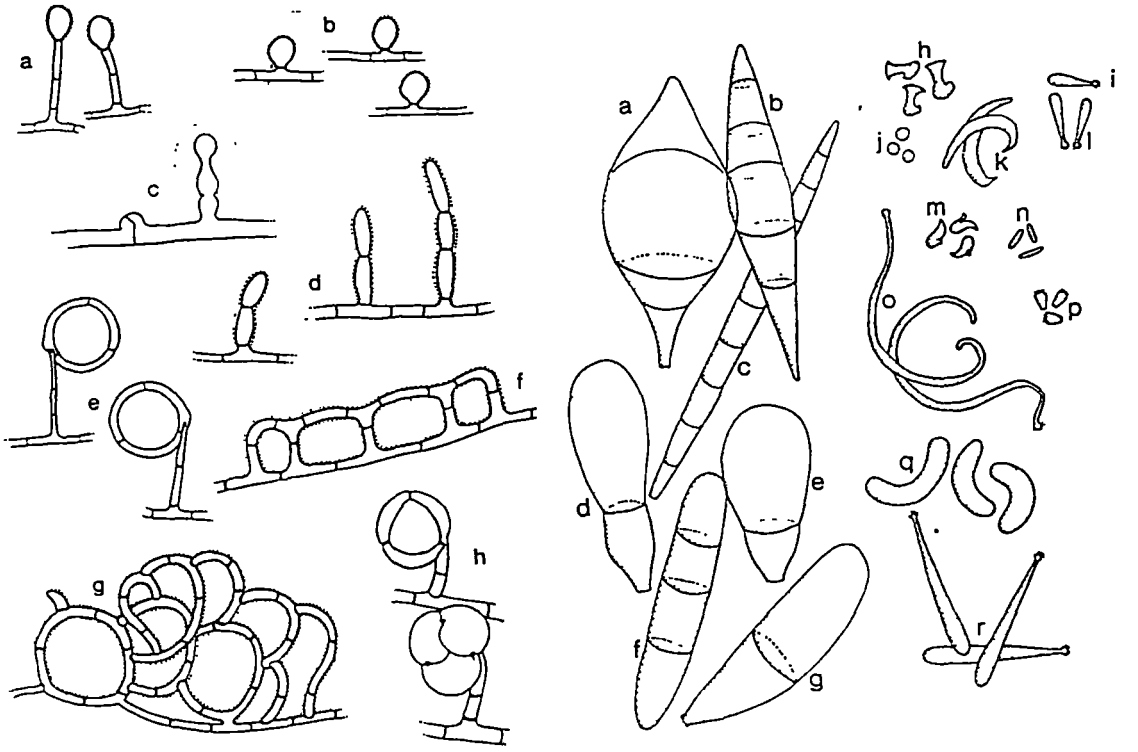
Predatory Fungi

The predatory fungi produce extensive hyphal networks in the environment. At intervals along the hyphae, trapping stuctures are produced for the capture of nematode prey. Captured nematodes are rapidly killed and penetrated by trophic hyphae. Traps come in a variety of forms; adhesive hyphae, in the form of branches, knobs, loops or networks; rings, either constricting or non-constricting; and sometimes a combination of rings and knobs. Some basidiomycetes release a toxin that paralyses passing nemas, which are then invaded by hyphae entering through the mouth and anus (Thorn and Barron, 1984).

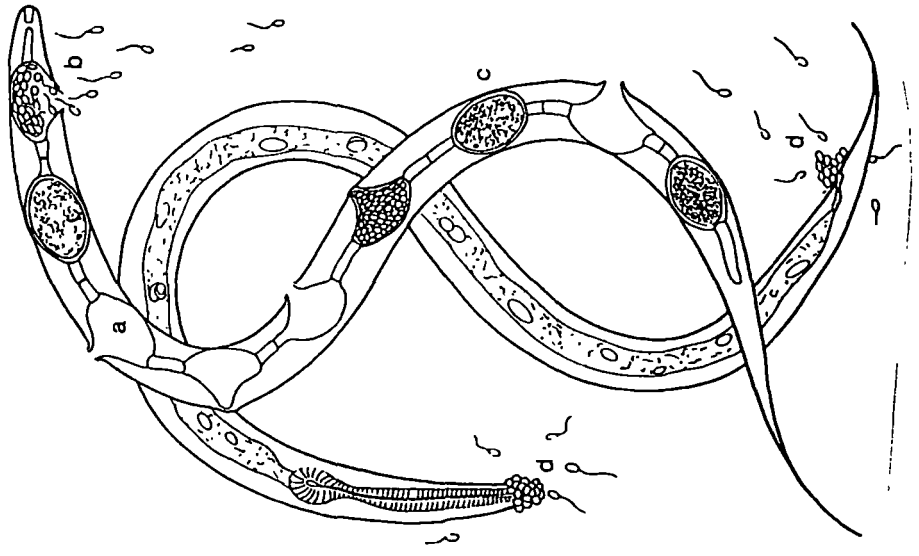
Entrapment in non-constricting rings occurs when a worm attempts to slide through a ring with a smaller diameter than that of its body. Often, more than one ring will be seen holding the captive, and some fungi have sticky knobs as well as rings to assist capture. If the struggling worm breaks free and swims away with the ring on its neck, it is killed later by trophic hyphae penetrating from the ring cells. Constricting rings trap their victims by rapid expansion of the three ring cells, closing the ring suddenly and very tightly on the victim's body.

The net-formers are the most commonly isolated of the nematophagous fungi (Barron 1977). They form short, curved hyphal branches which anastomose to form loops, usually in a

Fig. 3.4.1. Morphology of Nematophagous Fungi
Drawings from Barron (1977)



Above left:
Organs of capture in predatory fungi. a) stalked adhesive knobs. b) sessile adhesive knobs. c) hour-glass adhesive knob of *Nematoctonus*. d) adhesive branches. e) non-constricting rings. f) two-dimensional scalariform adhesive net. g) three-dimensional adhesive net. h) constricting rings.
Above right:
Spores of nematode-destroying fungi. a-g) predatory species. h-p) endo-parasitic species. q) predatory *Nematoctonus* species. r) endoparasitic *Nematoctonus* species.
Below:
The endoparasitic fungus, *Catenaria anguillulae*. a) empty zoosporangium. b) zoospores escaping through exit tube. c) resting sporangium in host body. d) zoospores encysting at orifices of host.



plane perpendicular to the substrate on which the fungus is growing. These loops can grow into complex three-dimensional networks, capable of trapping many nematodes at one time. The adhesive secreted on these networks binds specifically to nematode cuticle within moments of contact, and is strong enough to hold victims whether or not they have entered the net. The adhesive works even when the fungus is growing in water (Barron 1977), but does not require a water film for adhesion.

Endoparasitic Fungi

Those species of endoparasitic fungi whose conidia must be swallowed by the victim have evolved some remarkable conidial shapes (Barron 1977). These adaptations promote the lodging of conidia in the nematode's buccal cavity or pharynx. Other endoparasites produce adhesive conidia, less than 2 microns in diameter (*ibid.*), which attach to the cuticle of passing nematodes. *Catenaria anguillulae* (Chitridiomycetidae), is the most common of all endoparasites attacking nematodes. Flagellated zoospores differentiate in zoosporangia inside nematodes' bodies and escape through a single exit tube, actively swimming along chemical gradients to track down their prey. On reaching a host the spores encyst at or near body orifices and produce germ tubes which penetrate either through the orifice or directly through the cuticle. Perhaps the most sophisticated adaptation (*ibid.*) is found in *Haptoglossa heterospora*. This species develops in the host's body as one or more thalli, each of which produce several short exit papillae. Spherical non-motile spores are expelled through the tubes, and some remain in the sporangium. The spores germinate , producing another non-motile spore which is tongue-shaped (glossoid), and remains attached to the empty spherical spore, whose cytoplasmic contents have evacuated into the glossoid lobe. This contains an infection unit, held under tension, which is injected into the cuticle of any passing nematode.

3.5. Ecology

Nematophagous fungi are found in rotting wood, mossy places, leaf litter and decaying animal manure, and in a wide range of soils, worldwide (Barron, 1977; Mankau, 1980). Although some

can exist without nematodes in laboratory culture, all rely on nematodes to enable them to occupy their various ecological niches. Barron made some interesting observations concerning the isolation of predacious fungi from soil samples using the baited plate technique. Nematodes are added to an agar plate of weak maizemeal medium to which a light sprinkling of soil is added. The nematodes have a fungistatic effect on saprophytic fungi and stimulate the production of trapping organs on predatory fungi. Often the only fungi seen are predators.

Another technique (Dreschler 1941) uses plates previously overrun with *Pythium* or*Phytophthora* spp., to which a pinch of leaf mould or other organic debris is added. The succession of biota which ensues usually includes nematodes, which are subsequently decimated by predatory fungi, followed by another flush of nematodes which succumb to endoparasites. The petri plate ecosystem may well demonstrate the different roles that the nematophagous fungi have in the ecosystem at large. Barron suggested that the fast-growing net-formers are adapted to respond to a surge in nematode numbers, ceasing their activity when numbers dwindle, and the endoparasites, which tend to be more host-specific, are slower to respond and more likely to regulate the numbers of a particular nematode type. Using *Rhabditis terricola* as bait, Barron (1978) isolated 40 species of endoparasites from Ontario soils. These varied in their ability to parasitise bait nematodes which, Barron suspected, may have been due to differences in host specificity rather than variation in virulence. Barron (1977) suggested differences in prey specificity may be found in the predatory fungi if efforts were made to detect them. Mankau (1980) found a range of morphological, physiological and biochemical differences among 50 isolates of *A. conoides*, which also showed different degrees of predacity and response to trap-inducing factors. He suggested the taxonomy of these fungi requires a great deal of painstaking study.

Moisture

Soprunov (1958) grew mycelium of predacious fungi on chopped corn with a moisture content of 26-30% on a dry weight basis. For optimum sporulation he allowed the culture media to dry out. He noted a reduced efficiency in the ability of the fungi to reduce soil populations of pre-parasitic nematode larvae when the treated soils were given excess water. Transmission of the endoparasite, *Hirsutella rhossiliensis,* to cyst and root-knot nematodes, is inversely related to soil matric potential (Tedford, Jaffee and Muldoon, 1992). This the authors attributed to the production of fewer adhesive spores by the fungus in wet conditions. Predatory fungi growing from parasitised nematodes produced abundant traps when submerged in soil extracts but sporulated when growing in the atmosphere (Jaffee, Muldoon and Tedford, 1992).

Light conditions

Alternating light and dark and continuous light produced slightly greater growth than continuous darkness in fungi studied by Olthof and Estey (1965). Light stimulated sporulation and pigment production in the trapping fungi studied by Soprunov (1958).

pH

Olthof and Estey (1965) measured radial growth of 10 fungal species over a range of pH values. The fast-growing species grew over a wide range of pH values with optima at 5.0-6.0, while the slow-growing species grew best on media with an initial pH of 4.0. The pH of the media increased from 0.5 to 1.0 pH unit during growth in all species. *A. oligospora* had double optima at 5.0-6.0 and 7.0, with slightly less growth at 6.5. Soprunov (1958) reported a range from 6.0 to 7.5 as optimal for *A. oligospora* although conidia germinated and mycelium grew over a pH range from 4.0 to 9.0. The pH declined when predacious fungi were grown in a glucose solution, but increased if nematodes were added to the medium. The pH increased in a peptone medium, or when the fungi fed solely on nematodes. Grønvold *et al.* (1985) reported the pH optimum for *A. oligospora* was 6.0.

Temperature

The Turkmenistan fungal isolates studied by Soprunov (1958) had optimal growth temperatures from 28-33°C. This may be because they were more thermophilic than strains used by Olthof and Estey (1965) and Cooke (1963), whose estimates of temperature optima for radial growth of *A. oligospora* were between 22 and 30°C. Other species (*A. musiformis* and *A. superba*) grew best at 22°C, with a sharp decline in growth at 26°C. Pandey (1973) found 25°C optimal for *A. oligospora*, but his isolate grew poorly at 30°C and not at all at 35°C. Other species tested by Pandey grew well up to 35°C. The predaceous fungus studied by Larsen (Thesis, 1991), ceased growth at 5°C, but resumed when warmer conditions returned. Grønvold *et al.* (1985) held *A. oligospora* at 39°C for 23 hours and it resumed growth when returned to 23°C. This fungus remained fully viable after six days at -23°C.

Vertical Distribution in Soil

Gray and Bailey (1985) isolated nematophagous fungi at all depths down to 35cm in the soil from a deciduous woodland. The predators forming constricting rings, adhesive branches and adhesive knobs were restricted to the upper litter and humus layers, and the net-formers and endoparasites were found at all depths. The soils sampled had up to 10cm of leaf litter forming a species-rich, hemiedaphic zone, containing a greater density of nematodes than the underlying eudaphic zone in which fungal representatives were restricted to the net-formers and endoparasites.

5.6. Susceptibility of Nematode Species

Barron (1977) suggested susceptibility to the traps of *Arthrobotrys oligospora* could vary among nematode species, pointing out that free-living nematodes observed on soil plates after some weeks, seemed immune to the adhesive networks. He was unable to say whether this was due to staling products (by-products of fungal metabolism) affecting the adhesive properties of the nets, or whether the nematodes present were not

susceptible. It would not be surprising to find a degree of specialisation among nematophagous fungi, some being adapted to a particular host range. This is the case with the bacterium, *Pasturia penetrans,* (Sturhan 1988, Davies *et al,* 1988) and the fungus *Arthrobotrys dasguptae* (Boag *et al,* 1988).

Wharton and Murray (1990) found exsheathed J3s of *Trichostrongylus colubriformis* were not susceptible to the adhesive traps of *Arthrobotrys oligospora* and proposed that either the surface lectins were lacking on the J3 cuticle, or that the process of exsheathment had removed them.

Timper and Kaya (1989) found the sheaths of infective juveniles of entomopathogenic nematodes protected them from endoparasitic fungal spores but not from the adhesive traps of predatory fungi. They also noted that sometimes trapped larvae escaped by shedding their sheath before they were infected. Generally, however, ensheathed juveniles remain fastened to adhesive traps until penetration processes result in their death.

Waller and Faedo (1993) assessed the predacity of four *Arthrobotrys*, two *Geniculifera* and two *Monacrosporium* species against infective juvenile *Trichostrongylus colubriformis*, *Haemonchus contortus* and *Ostertagia circumcincta* larvae. The nematodes were susceptible to all fungal species, but there were differences between nematode species in the proportion of their population removed by predation, though these were not statistically significant. *O. circumcincta* were the most susceptible and *T. colubriformis* the least susceptible of the nematode species. *Geniculifera eudermata* removed over 80% of all species and over 90% of *H. contortus* and *O. circumcincta.* This study showed that species other than the commonly studied *A. oligospora* have potential as biocontrol agents against animal-parasitic nematodes.

3.7. Dependence on Nematodes

Many nematophagous fungi will grow saprophytically in the absence of nematodes, but their slow growth rates make them

poor competitors in the presence of other soil microorganisms. The presence of these fungi in a soil sample can be detected by incubating samples on very dilute nutrient media with bait nematodes added (Wyborn *et al.*, 1969). Observations must then be extended over several weeks if all types of fungi are to be detected.

3.8. Nutrition

Most of Soprunov's (1958) predatory fungi were capable of fermenting glucose, and fewer fermented lactose, maltose or sucrose, but none were capable of attacking cellulose or lignin. *A. oligospora* , *A. conoides* and *Monacrosporium cystosporum* utilised almost all of the 18 carbohydrate and nitrogen sources tested by Saxena *et al.* (1989), and for good growth required thiamin, biotin and para-4-aminobenzoic acid. Blackburn and Hayes (1966) also noted a response to thiamin, biotin and PABA but these were not essential for growth of *A. oligospora* and *A. robusta*. A wide range of substances supported growth in these fungi, with highest yields of mycelium coming from carbon sources having the same configuration as glucose around carbon atoms, 3, 4, 5 and 6 (*ibid.*). Oligo- and polysaccharides in general could be utilised. Cellobiose, an intermediate in the degradation of cellulose, produced the highest mycelium yield, and cellulose was easily degraded. Nitrogen requirements could be adequately fulfilled by inorganic or organic sources. Blackburn and Hayes (1966) concluded that the fungi have simple nutrient requirements and appear to gain no benefit from the ability to catch eelworms other than the provision of an alternative source of energy.

3.9. Growth Rates

Olthof and Estey (1963) classified the fungi into three categories with regard to growth: the fast-growing, the intermediate-growing and the slow-growing species. *A. oligospora, A. conoides, A. musiformis* and *A. robusta* belong to the fast-growing group; *A. superba, Dactylella ellipsospora and D. asthenopaga* are in the intermegiate category, whereas *A. dactyloides, D. gephyropaga* and *D. brochopaga* are slow-growing species. Pandey (1973)

agreed with Olthof and Estey, finding the net-forming species to be the fastest-growing. Species producing adhesive knobs, adhesive branches and constricting rings were, in general, slow growers.

3.10. Predacity

Cooke (1962, 1963a, 1963b, 1964) investigated the ecology of nematode-destroying fungi in order to determine methods whereby the most efficient predators could be selected for use as biocontrol agents of plant-pathogenic nematodes. He found that the fungi could be categorised into three groups,
(i) the net-formers, which form traps only after induction,
(ii) the adhesive knob and ring formers which form traps spontaneously, and
(iii) the endoparasites.
After a series of experiments using soil or sand microcosms in which various fungi were pitted against free-living nematodes, he was able to rank these groups (i)<(ii)<(iii), in their comparative efficiency in reducing nematode numbers. His conclusions were supported by the findings of Blackburn & Hayes (1966) and Jansson (1982).

Cooke reasoned that as trapping became more efficient, by evolutionary processes, fungi would have less need to compete as saprophytes. He expected to find the more vigorous saprophytic growers to be less efficient predators than those which relied on predation alone for survival. He measured efficiency by counting the nematode population present after a certain incubation time (Cooke, 1963a). The relatively rapid-growing net-forming fungi provided food for the nematodes, and frequently Cooke found higher numbers in the fungal treatments than in the control plots. He clearly demonstrated that these fungi do not destroy populations of free-living soil nematodes.

Hans-Börje Jansson (1982), using the nematode, *Panagrellus redivivus* as bait, ranked the fungi in order of predacity, with results which agreed with those of Cooke. He found predacity to be correlated to the attractiveness of the fungi to nematodes, and found the trap-bearing hyphae of *Arthrobotrys oligospora* were

more attractive than its non-trapping hyphae. Heintz (1978) devised a predatory index, based on the number of days needed for a fungus to eliminate a standard nematode population on a petri plate. Her results varied somewhat from Cooke's, which she suggested indicated differences in predacious efficiency within species. She found no correlation between growth rate and predacity, in contrast to Cooke (1963), Hayes and Blackburn (1966) and Jansson (1982), who suggested increasing predacity would be accompanied by loss of saprophytic ability.

Methods for Estimating Predacity

Predacity has been estimated by four different methods; the agar disc method, soil or sand microcosms, the baited petri plate method and the faecal culture or dung pat bioassay.

The Agar Disc Method

Cooke (1962) developed an agar disc method in which slides bearing agar discs were buried in soil for a period and later examined for trap-bearing hyphae. Predacity was estimated from the number of traps per disc.

Soil and Sand Microcosms

Hayes and Blackburn (1966) used a method similar to that later adopted by Jansson (1982). Their experiments on nutrition of the predaceous phase were carried out in sand culture vessels containing 20g sand. Fungal spores were included in 5 ml aliquots of nutrient solution averaging 30 spores per ml. After 4 days' incubation nematodes were added in 0.5 ml water at the rate of 100 per vessel. The nematode used was *Panagrellus redivivus*, a free-living nematode cultured on oatmeal in the laboratory. After 5 days' incubation, nematode populations were counted, and the numbers found in vessels containing only sand and nutrient solution,(A), together with the numbers in four fungus-inoculated vessels, (B), were used to determine the "predacity number" = $(A-B)/A$ * 100. The nutrients stimulated nematode reproduction to different degrees, and the final nematode populations were five to eight times the inoculum numbers.

Jansson (1982) used 30g quantities of loam rich in organic matter in his experiments. He considered that the organic content (10.5% dry weight) was necessary to widen the range of water contents so that both nematodes and fungi could do well. Soil was autoclaved in 100ml Erlenmeyer flasks, and 1 ml of a macerated fungal culture suspension was added as small drops. After six days' incubation to establish the fungus, 3,000 *Panagrellus redivivus* in 1 ml of water were added per flask. Two or three 5 g samples of wet soil were taken from three flasks (six or nine replicates) at time intervals and the nematodes extracted and counted to estimate predacity = 100 * (C-F)/C, where C is the mean number of nematodes in control flasks and F is the number in fungal flasks. This equates to the predacity number of Hayes and Blackburn.

Baited Petri Plates

Pandey (1973) added infective larvae of *Trichostrongylus axei* and *Ostertagia ostertagi* to corn meal agar cultures of ten nematophagous fungi and estimated trapping efficiency by counting the number of larvae attacked and immobilised among 100 observed after various time intervals.

Heintz (1978) added 2,500 *Panagrellus redivivus* or *Aphelenchoides rutgersi* to corn meal agar petri plate cultures of fungi and used the number of days until all worms were removed as a measure of predacity. For each of ten sets of experimental conditions, a P value was determined and the predatory index (PI) of each fungus was found from the sum of its P values.

Faecal Cultures and Dung Pat Bioassay

Larsen (Thesis, 1991) compared the use of faecal cultures and dung-pats for assessing the ability of nematophagous fungi to reduce pre-parasitic nematode numbers. The mixing of vermiculite with faeces provides better aeration in faecal cultures than can be expected in dung pats, which enhances the growth of both fungi and nematodes. The dung pat, on the other hand, provides a more realistic environment for the assessment

of the control agent, and yielded from only 38% to 81% the number of larvae obtained from faecal cultures in the absence of fungus.

Problems with Predacity Estimates

Cooke (1963a) frequently found the addition of net-forming fungi to soils increased the number of free-living nematodes. He used the naturally occurring nematodes in the test soil as bait, and their numbers were low (about 5 per g.) before adding fungal inoculum. The increase in nematode numbers was correlated to the amount of inoculum added and was clearly the result of improved nematode nutrition. The fungi were grown in 2% maize meal sand cultures and this was used as inoculum at rates of 2 to 15% by weight. The ring-forming fungi grow slower than the net-formers, and would not have provided sufficient mycelium for feeding nematodes. Nematode numbers declined with these fungi but increased ten or twenty fold with the net-formers.

Cooke (1963b) also included saprophytic ability in a competitive soil environment in his ecological investigations. He found the net-formers had higher growth rates and were more competitive than the adhesive branch, knob- and ring-forming fungi and suggested that the evolutionary development of predacious efficiency has been accompanied by a loss of rapid growth rate and competitive saprophytic ability. However, the use of free-living nematodes, which include bacterivorous and fungivorous species, has complicated his results and those of all other investigators who used such bait.

Jansson (1982) pointed out that the nematodes have a dual function in soil microcosms, as food for the fungi and as the stimulus for trap induction. It seems likely that the numbers present in Cooke's soils were below the threshold of stimulus for some of the fungi tested. Jansson supported Cooke in his conclusions, yet there are internal inconsistencies in his results. He found that 6 days of incubation before the addition of nematodes produced a peak predacity of 30% in *A. oligospora,* and that this level was reached after 10 days incubation with nematodes. However, in other experiments conducted under

similar conditions, different estimates of predacity were obtained. He compared the predacity of *A. oligospora* and *Dactylaria candida* over a 32 day period, again after 6 days incubation before the addition of nematodes. After 7 days, both fungi were almost equally predacious at about 80%. In a third experiment he rated the predacity of *A. oligospora* at 70%.

Blackburn & Hayes studied the nutrition of two *Arthrobotrys* species in both the saprophytic (Blackburn and Hayes 1966) and the predacious (Hayes & Blackburn 1966) phases. Their results (see fig. 3.10.1.) further illustrate the problems inherent in using this method to estimate predacity.

On the basis of the results obtained, Hayes and Blackburn concluded that ammonium nitrogen and amino acids suppressed trapping efficiency in *A. oligospora.* Trapping they conjectured, may reflect a response to an altered C/N ratio (the need for supplementary N stimulating trapping or the supply of excess N suppressing trapping) rather than the existence of a direct predator/prey relationship between nematode and fungus.

If their results are scrutinised, it is apparent that ammonium nitrate produced the highest yield of nematodes in the absence of fungus (the controls), and *Arthrobotrys oligospora* reduced this by more than fifty percent, trapping an average of about 460 nemas per vessel. This figure is comparable to the numbers caught in the potassium nitrate and sodium nitrate treatments, yet they scored predacity numbers of 92 and 91 while ammonium nitrate scored 56. The differences here arose from the effects of the nutrient medium on nematode reproduction, rather than its effects on fungal trapping behaviour. Because ammonium stimulated reproduction more than the nitrate solutions did, final nematode numbers were higher. Asparagine was similar to ammonium nitrate in stimulating nematode reproduction, but produced the greatest reduction in nematodes of any treatment, about 730 being removed by trapping. This resulted in a predacity number of 89, not a true reflection of the

Table 3. *The effect of nitrogen source on growth and predacity of two nematode-trapping fungi*

Nitrogen source	Mean mycelium yield in mg. dry wt.		Mean of nematode populations obtained from four fungus-free sand cultures	Mean of nematode populations obtained from four inoculated sand cultures		Predacity number	
	A. oligospora	*A. robusta*		*A. oligospora*	*A. robusta*	*A. oligospora*	*A. robusta*
Control: no nitrogen	28·9	30·9	468 (13·4)	453 (14·2)	452 (10·3)	—	—
Potassium nitrate	281·0	309·3	504 (10·9)	40 (7·2)	651 (13·2)	92	−29
Sodium nitrate	304·1	323·2	563 (23·1)	49 (5·7)	839 (20·5)	91	−49
Sodium nitrite	318·1	56·7	452 (16·6)	72 (9·2)	208 (23·4)	84	54
Ammonium nitrate	299·8	356·6	823 (18·9)	359 (14·8)	1210 (42·2)	56	−42
Ammonium sulphate	321·1	363·3	517 (13·9)	423 (8·5)	649 (30·4)	18	−26
Ammonium chloride	310·4	363·7	630 (13·8)	466 (16·5)	715 (13·9)	26	−14
Asparagine	385·1	304·4	821 (15·0)	90 (4·9)	1028 (41·9)	89	−25
Urea	308·7	372·4	580 (29·9)	150 (37·9)	619 (22·9)	74	−7
Mixture of amino acids	327·4	303·9	494 (28·7)	385 (21·3)	715 (14·7)	22	−45

Standard deviations of means are given in parentheses and have been corrected for bias.

Fig. 3.10.1. Table of data from which Hayes and Blackburn concluded that ammonium nitrogen reduced the predacity of *A. oligospora* (from Hayes and Blackburn 1966)

increased trapping activity. The reason for this treatment producing the highest capture rate may be that it resulted in more complete colonisation of the sand, a conclusion which is supported by the fact that asparagine produced the greatest mycelium weight of any nutrient treatment.

The other problem with these authors' conclusions is that they failed to take into account the stimulatory effect of the fungus itself on the nematode population. Some idea of the magnitude of this may be gained from of the numbers of nematodes recovered from the vessels inoculated with *Arthrobotrys robusta* , which scored negative predacity numbers for most treatments. (Perhaps this illustrates the weakness of this experimental method; obviously this fungus trapped some nematodes and would be more predaceous than a non-trapping saprophyte.) Substituting these figures for the controls, it seems likely that *A. oligospora* may have reduced the population by 850 in the ammonium nitrate treatment, rather than by 460 as the controls would suggest. Even so, the resulting predacity number would have been 70, still not a true reflection of the increase in capture numbers. In this case, even though there was twice as much trapping activity occurring in these vessels, the authors concluded that ammonium nitrate reduced the effect of the fungus. That the early larval stages of nematodes are less likely to be trapped than larger, older ones, further complicates the picture, and may help explain the results. It is from work such as this that the net-forming fungi have come to be considered inefficient predators, unlikely to be of use as biological control agents. The belief that net-forming fungi are relatively inefficient at reducing nematode numbers has persisted; for example, Sayre and Walter (1991) cited Jansson's work in their review of factors affecting nematode populations.

However, for reducing numbers of pre-parasitic larvae in animal manure, the net-formers were the most effective of the predatory fungi Larsen (Thesis, 1991) tested. The predatory fungi were also the most effective of those tested by Soprunov (1958) and Pandey (1973). This may reflect the faster growth rates of these fungi, which would give them a better chance of

colonising faeces. The pre-parasitic larvae used to estimate predacity in these cases were non-reproductive and thus did not complicate the results.

In further studies, Larsen, Wolstrup, Henriksen, Dackman, Grønvold and Nansen (1991) incubated soil and compost samples in dilute rumen fluid before isolating nematophagous fungi. After further screening the isolates by subjecting them to simulated gastro-intestinal conditions, dung-pat bioassay against *Ostertagia ostertagi* showed that net-forming species of the genera, *Arthrobotrys* and *Duddingtonia,* were the most efficient predators of animal-parasitic nematode larvae.

3.11. Obligate and Facultative Predators

Certain predatory fungi form traps spontaneously when growing in pure cultures, while others require induction before trap formation will occur. This has led to the concept of obligate and facultative predators, and the suggestion that the obligate predators would be superior candidates for biocontrol agents (Cooke 1962). Mankau (1962) noted that conidia of some trapping fungi formed traps immediately on germination in certain soils. More recently it has been demonstrated that some "facultative" predators exhibit spontaneous trap formation when their spores germinate in manure (Dackman and Nordbring-Hertz, 1992) or in aqueous extracts of certain soils (Jaffee *et al.,* 1992). Jaffee *et al.* (1992) found that the net-forming fungi produced traps when growing from parasitised nematodes in saturation extracts of soils, and that the facultative predator status assigned to them by Cooke may underestimate their ecological significance. Observations of trap formation must be made under laboratory conditions, and for this reason the "obligate" and "facultative" distinction may be artificial and misleading.

3.12. Trap Induction and Capture Processes

The formation of traps has been studied particularly thoroughly in the case of *Arthrobotrys oligospora*, and the physical and

chemical processes involved in capture, death and consumption of nematodes are now quite well understood.

Chemical Stimuli

The discovery that sterile filtrate from nematode cultures induced trapping, as did melted snow, weak solutions of ethanol or sodium hydroxide (Sopranov 1958), and various materials of animal origin led Pramer and Stoll (1959) to propose the term "nemin" to describe the activating substance or substances. Various workers have contributed to elucidating the nature of nemin, culminating in the work of Nordbring-Hertz and Brinck (1974). Trap-inducing compounds were found to be peptides with a high proportion of non-polar and aromatic amino acids, including leucine, isoluecine, valine, proline and tyrosine. Carbon dioxide is essential for trap induction but species vary in their response to its concentration (Bartnicki-Garcia *et al*, 1964). Schenck and Pramer (1975) could find no evidence that volatile compounds from nematodes were involved, but Nordbring-Hertz (1977) suggested that ammonia is excreted by nematodes in amounts which could affect trap induction. Soprunov (1958) suggested that the reaction of ammonia with carbon dioxide to form ammonium carbonate may be involved in trap induction.

The importance of mineral salts to trap induction was demonstrated by Nordbring-Hertz and Brinck (1974). On weak cornmeal agar, no traps were induced by the phe-val dipeptide in *Arthrobotrys oligospora*, unless the agar was prepared with a supplement of mineral salts. The later discovery (Dackman & Nordbring-Hertz, 1992) of spontaneous trap formation by conidia of *Arthrobotrys oligospora* germinating in manure, supports the hypothesis of Jaffee *et al*, (1992), that behaviour of nematode-trapping fungi on agar plates may differ from behaviour in the soil. Veenhuis *et al.* (1989) dissected trapped nematodes from the surrounding mycelium and placed them in distilled water to study hyphal development free of environmental influences. Hyphae only arose from trap cells, and never from trophic hyphae. These hyphae were always vegetative; no trapping hyphae were seen growing from parasitised nematodes. In contrast to their observations, Jaffee *et al.* (1992) found that

traps were produced from parasitised nematodes held in soil extracts and in 2 mM KCl. The number of traps produced varied between soils, and was greater in soil extracts than in 2 mM KCl.

Nematode Stimuli

Living nematodes induce trap formation in *Arthrobotrys oligospora* more rapidly than morphogenic peptides, and Nordbring-Hertz (1977) suggested there were factors other than peptides involved in nematode-induced trap formation. found there was The threshold level of nematode density required to elicit the trapping response on baited plates, varies between fungal species (Wyborn *et al.* 1969). To ensure stimulation of all species, 5,000 nematodes were required (Wyborn *et al.* 1969). *Arthrobotrys oligospora* was the most responsive of the fungi tested, with a threshold of 1,000 nemas per plate.

Variation in Response to Inductive Stimuli

Lysek and Nordbring-Hertz (1981) suggested that the endogenous rhythm of trap formation they observed by may be an adaptation that permits the fungus to vary its response to the presence of nematodes as their population size changes. It would not be advantageous to the predator to exterminate its prey and the reduction in trapping after the initial flurry of activity (Barron, 1977), may be an expression of altered sensitivity to inductive stimuli. Barron reviewed the research on trap induction from the discovery in 1937, of nematode-induced trap formation in *Dactylella bembicoides,* onwards. He pointed out that the apparently conflicting results obtained by different workers was probably a consequence of variation in the responsiveness of different species to inductive stimuli.

Adhesive knob formation by conidia of *Drechmeria coniospora* is suppressed by increasing conidial density (van den Boogert *et al,* 1992). This endoparasitic fungus produces clumps of conidia sequentially on conidiophores extending from their victims' bodies. As conidia mature, they produce an adhesive knob which will fasten to the cuticle of any passing nematode. Maturation is delayed in most of the conidia in a clump until dispersal occurs,

either by attachment of mature conidia to a passing nematode, or disturbance of the soil by other animals or processes. This aids the dispersal and persistence of the fungus, which is entirely dependent on nematodes for survival. Nematodes are not required for sticky knob production, and their presence does not induce knob production in immature conidia. The function of sticky knobs in this fungus is transmission between hosts, whereas the traps of predaceous fungi have a more direct role in supplying nutrients to the colony.

Trap and Adhesion Processes

Jansson (1982) showed that nematodes are attracted to mycelium of trapping fungi and that this effect is enhanced when traps are present. The adhesive secreted on the trapping structures of fungi is a polymer containing proteins and carbohydrates (Tunlid *et. al.*, 1991), both of which are involved in binding to the nematode cuticle in a lectin-mediated interaction (Nordbring-Hertz & Mattiasson, 1979).

Barron (1977) produced a series of micrographs and diagrams showing the capture organs, invasion processes and spore production of many forms of predatory and endoparasitic nematophagous fungi. Similar diagrams have been drawn by Soprunov (1958), Haard (1968), Fowler (1970), Jarowaja (1970), and Pandey (1973). Nordbring-Hertz (1977) produced a series of micrographs showing trap formation in *A. oligospora* and Veenhuis *et al.* (1989) used light microscopy and video enhancement to provide excellent images of the events following capture.

Penetration Processes

Murray and Wharton (1990) described the processes involved in the capture and invasion of all the free-living stages of *Trichostrongylus colubriformis* by *Arthrobotrys oligospora*. After the initial binding between the fungal adhesive and the nematode cuticle in first and second stage larvae, there was a time lapse of 15 minutes before the production of an infection bulb. This expanded under the cuticle, reaching its maximum

diameter after 1 hour. Invasive hyphae were generated 1.5 hours after capture, and grew through the body until it was filled, 12 hours after capture.

Third stage larvae are somewhat protected by the L2 sheath, and the infection process took twice as long. Trap hyphae in contact with the nematode swell, causing a small depression in the sheath. At this point, a penetration hypha forms and forces its way through the sheath, creating a primary infection bulb. A number of secondary infection bulbs may be formed under the sheath before the invasion of the L3 cuticle by a secondary infection bulb. Invasive hyphae then proceed to absorb the internal tissues.

The time from capture to penetration of the sheath took 2.5 hours, with a further 3.5 hours before invasive hyphae could be seen. The whole process took an additional 12 hours to reach the stage where invasive hyphae completely filled the body cavity, compared to L1s and L2s. Murray and Wharton produced 3D reconstructions of the capture and penetration processes from scanning electron microscope images. They suggested that cessation of movement was due to loss of turgor in the nematode, rather than a toxin. However, Olthof and Estey (1963) showed that filtrates from nematodes parasitised by *A. oligospora,* contained a nematode-inactivating substance.

Veenhuis *et al,* (1989) studied the fate of electron dense microbodies in the trap cells of *Arthrobotrys oligospora* during the initial hours following capture of a nematode. The trap cells contain numerous such microbodies, but these rapidly decrease in the cell forming the penetration peg invading the nematode. After 1-2 hours the infection bulb is fully formed and trophic hyphae formation begins. By this time the trap cell has all the organelles of a normal vegetative cell and no dense bodies. These appear to degrade and eventually form vacuoles. Dense microbodies are probably energy storage devices which contribute to the survival of the trap cells and which provide energy for rapid penetration of nematode prey.

3.13. Nematotoxin Production by Nematophagous Fungi

Nematotoxins are released to the environment by the hyphae of some predatory fungi (Barron and Thorn, 1987; Cayrol *et al.*, 1989; Giuma and Cooke, 1971; Larsen, 1991) and by the germinating conidia of some endoparasitic fungi (Giuma, Hacket and Cooke, 1973). In *Arthrobotrys oligospora* however, production of toxin is restricted to the trophic hyphae within the victim (Olthof and Estey, 1963). The agent may be ammonia (Balan and Gerber 1972), or toxic polysaccharides (Giuma *et al.*,1973). Waller and Faedo (1992) found many fungi produced nematicidal substances in response to the presence of nematodes, but the potency of these was not high enough to warrant isolation and identification.

3.14. Nematophagous Fungi as Potential Biocontrol Agents

Duddington (1957) and Barron (1977) provided good reviews of the history of biological control attempts using nematophagous fungi. The classic work of Linford and his colleagues was the first time predaceous fungi were shown to be capable of reducing crop losses from disease caused by nematodes (Linford and Yap, 1938, 1939; Linford, Yap and Oliveira, 1938). The effect relied on green manuring to stimulate both the fungi and the soil nematode population, the resulting flush of predatory activity accounting for high mortality in the pathogenic nematode population. Duddington (1957) also considered an organic supplement was necessary, finding that unless manure was added to the soil with the inoculum of trapping fungi, control of potato eelworm was not obtained.

Mankau (1980) reviewed progress in the fungal control of nematodes. He emphasised the difficulties inherent in introducing an organism to the average agricultural soil which contains approximately 10^9 bacteria, 10^{5-8} actinomycete, 10^{5-6} fungus, and 10^{4-5} protozoan reproductive units per gram, plus a large number of fungivorous nematodes, tardigrades, Collembola, mites, and other assorted microfauna and meiofauna. Predacious fungi tend to be poor saprophytic competitors and many soils are fungistatic to them (Mankau, 1962). However, he saw cause for

optimism in the use of commercial *Arthrobotrys* isolates to control *Ditylenchus myceliophagus* in commercial mushroom production and *Meloidogyne* in tomato crops. He considered the egg-parasitic fungi had potential and reported a case of natural control of *Meloidogyne* in peach orchards by the fungus *Dactylella oviparasitica* .

Sayre and Walter (1991) updated this review with the information that *D. oviparasitica* was unable to control *Meloidogyne* on the roots of tomato and grapes because it could not keep pace with the higher reproductive rate of the nematode on these more favourable host plants. They reported the findings of Jaffee and Muldoon (1989) that the nematophagous fungus, *Hirsutella rhossilienses*, was responsible for the natural supression of *Heterodera schachtii* on cabbage roots.

Stirling (1989) was able to control *Meloidogyne incognita* on ginger roots with organic amendments which stimulated fungal activity. Stirling and West (1991) screened 26 isolates of *Paecilomyces lilacinus* and 13 isolates of*Verticillium chlamydosporum* for activity against eggs of *Meloidogyne javanica* and selected three isolates which were highly parasitic. The two *V. chlamydosporum* isolates will be used in further tests but health and safety considerations may preclude the use of *P. lilacinus* as a biocontrol agent. This fungus may be pathogenic to humans.

3.15. Examples of Biocontrol using Fungi

Renewed interest in the use of fungi in controlling plant pathogens and weeds has resulted in the development of production techniques and formulations which enhance fungal performance. Liquid culture of the endoparasitic fungi, *Drechmeria coniospora, Verticillium balanoides* and *Harposporium anguillulae* has been described (Lohmann and Sikora 1989), and the use of different animal manures or wheat grains in the production of nematode egg parasitic fungi affects the ability of these fungi to control galling caused by *Meloidogyne javanica* on tomato plants (Abu-Laban and Saleh

1992). *Colletotrichum truncatum, Alternaria cassiae* and *Fusarium lateritium* have been encapsulated in pasta for application as mycoherbicides (Connick, Boyette and McAlpine 1991), and an invert emulsion has been developed to enhance germination of mycoherbicides sprayed onto the leaves of target weeds (Connick *et al.* 1991, Boyette *et al.* 1993). The addition of acid to a vermiculite carrier prevents bacterial degradation of fungi formulated to control damping-off disease (Lewis *et al.* 1991) and alginate-encapsulated mycelia may be used in mass-production of fungal spores (Daigle and Cotty 1992). *Meloidogyne* control agents have been formulated in powder (Gomes-Carneiro and Cayrol 1991), and a commercial crown-gall control agent is formulated on peat (Ryder and Jones 1990). Effective control of *Meloidogyne* on kiwifruit was achieved by adding a commercial formulation of *Arthrobotrys irregularis* T-350 to composted mulch at the time of application to the trees (Cayrol *et al.* 1991).

3.16. Biocontrol of Animal-Parasitic Nematodes

The nematode parasites of ruminants spend the first part of their lives as free-living nematodes (see chapter 2). This brings them into contact with many biotic and abiotic factors with the potential to devastate their population numbers. The free-living phase is also the means of transmission to new hosts as it culminates in the infective larval stage of the parasite. The biological investment in producing these is necessarily large, as abiotic factors such as high temperatures and drought frequently cause up to 100% mortality in larval cohorts. When conditions for development are favorable, however, enormous numbers of infective lavae may survive, causing epidemics of helminthosis, as well as chronic ill thrift and production losses.

Biotic mortality factors of the free-living stages include bacterial and viral diseases and predation by various organisms including collembolla, free-living nematodes and fungi. Of these, the fungi appear to offer the greatest hope as biological control agents (Waller & Larsen 1992).

3.17. The Case for Biocontrol

There are several reasons for seeking a biocontrol agent.

1. Some farmers wish to minimise the use of chemicals. Heightened environmental awareness has produced a demand for residue free products and stricter control of drug usage.
2. Drench resistance is increasing in worm populations.
3. Effective chemicals are costly to research and develop and may become increasingly rare. Alternative management practices are necessary to augment the use of chemotherapy in order to delay the development of resistance.
4. The result of biocontrol would be prevention, rather than cure, of helminthosis epidemics.
5. Ideally, an effective biocontrol agent, once established, would reduce both the incidence of epidemics and the need for chemotherapy, increasing productivity and reducing costs for the farmer.

Waller (1992) suggested Integrated Pest Management (IPM) incorporating grazing management, the use of anthelmintics, and the utilization of natural or artificially induced immunity, would constitute a sustainable system for controlling nematode parasites. He stressed the need to change the attitudes of producers who have historically relied on chemotherapy, in controlling parasitic nematodes.

Waller's suggested IPM techniques for nematode control include better use of existing drugs, development of helminth vaccines, breeding for worm resistance, development of new anthelmintics or alternative compounds, and biological control. In reviewing prospective biocontrol agents, Waller (1992) considered the nematode-trapping fungi to offer the greatest initial hope of success.

It is during the free-living stages that larvae are susceptible to trapping fungi. The susceptibility of larvae of *Trichostrongylus, Haemonchus, Ostertagia* and others, has been demonstrated (Wharton & Murray 1990, Gronvold *et.al.*,1985, Nansen *et.al.*,1986,88, Pandey 1973, Deschiens 1939,Descazeaux 1939).

Environmental conditions which are most suitable for the development and survival of the free-living stages of nematodes on pasture are also the most suitable for fungal activity (Waller and Larsen, 1992).

Laboratory cultures of nematophagous fungi are able to destroy large numbers of infective-stage nematode larvae within days. Reports of greater than 90% reduction in nematode numbers are common (Sopranov 1958, Hayes & Blackburn 1966, Pandey 1973, Jansson 1982, Grønvold *et al*, 1985, Nansen *et al*, 1986, Larsen 1991), and if this could be repeated in the field, then fungi could offer great hope for reducing the incidence of epidemic helminthosis in young livestock such as calves and lambs. The potential for nematophagous fungi to control free-living stages of parasitic nematodes has been recognised for decades, but research efforts have not yet succeeded in establishing the practicality of this approach.

Larsen (1991) discussed the ecology of the dung pat as an environment for development of nematode larvae and fungi. In cattle dung, which tends to have a high water content, microbial activity removes oxygen from the inner regions causing anaerobic conditions, unsuitable for fungal growth. Larsen considered the region between the crust and the anaerobic region to be the site of fungal growth. The fresh dung pat offers early colonisers a rich substrate relatively free from competition and fungistatic factors likely to exist in the underlying soil. As such, it is an ideal environment for establishment of nematophagous fungi. In his "Studies on the capability of microfungi to destroy animal-parasitic nematodes", Larsen (Thesis, 1991) found the net-formers were the most efficient predators of all, although he did not rule out the possibility that endoparasites may become useful if the problems involved in their mass production could be overcome. The most effective fungi Larsen tested were a strain of *Arthrobotrys oligospora* and a strain of *Duddingtonia flagrans*, the latter of which produced chlamydospores capable of surviving passage through ruminants.

Waller and Larsen (1993) reviewed progress towards utilising predaceous fungi against gastro-intestinal nematodes in livestock. The potential for success in controlling these nematode pests may be greater than has been experienced in attempts to use these fungi to control plant-parasitic nematodes.

3.18. Attempts at Biocontrol of Animal-Parasitic Nematodes

The earliest report of the susceptibility of pre-parasitic larvae of animal-parasitic nematodes to nematophagous fungi came from French scientists (Roubaud and Deschiens,1939; Roubaud and Descazeaux, 1939), who found that *Dactylella ellipsospora* could capture larvae of *Strongyloides* and *Ancylostoma* species. These workers and their colleages produced a series of publications (Descazeaux, 1939a, 1939b; Descazeaux and Capelle, 1939; Deschiens, 1939a, 1939b, 1939c, 1941, 1942, 1943; Deschiens and Lamy, 1943). Their work culminated in a small field trial in which a $25m^2$ plot was treated with a mixture of *D. ellipsospora* and *Arthrobotrys oligospora* spores prior to grazing by two lambs. These lambs developed fewer *S. papillosus* and *Bunostomum* parasites than did two lambs grazing an untreated plot (Roubaud and Deschiens, 1941a, 1941b).

From 1954 to 1956, Soprunov (1958) spread a powdered preparation of the spores of six predatory fungi in a coal mine at the rate of 100 to 150 g/m^2. The incidence of ancylostomiasis in the miners was 38.5% in the spring of 1954 when treatment commenced and fell to 8.6% by autumn 1956. Since there was no change in the incidence in other, untreated mines during this period, Soprunov felt justified in claiming to have proven the ability of nematophagous fungi to control the disease by reducing the survival of infective larvae.

Pandey (1973) studied the activity of 10 predatory fungi against the larvae of *Trichostrongylus axei* and *Ostertagia ostertagi* on agar plates. The larvae were susceptible to the traps of all species, although the adhesive knobs of *Monacrosporium ellipsospora* were only moderately effective. The net-forming

fungi were the most efficient predators and the knob- and ring-formers the poorest according to Pandey's results.

Hashmi and Connan (1989) presented the results of unpublished work in a review article. They found that the conidia of *Arthrobotrys oligospora*, added to faecal cultures containing eggs of *Cooperia oncophora* at the rate of 20/g, reduced the yield of larvae over three weeks by only 40%, but when the rate was increased to 50/g, yield of larvae was reduced by 86% over four weeks. They claim to have reduced the density of infective larvae on pasture by up to 72% by mixing *A. oligospora* conidia into artificial cow pats and sheep faeces. They stated that "*A. oligospora* is passed in the faeces of cows throughout the grazing season with a peak in spring and late summer", and that "when conidia was fed to a housed calf, the fungus was passed in the faeces for the next five days." In a further field trial calves were orally dosed with conidia at the rate of 8 million twice weekly, but the reductions in pasture contamination were disappointing. The authors concluded that although the dose rates were high, the amount of fungus passed in the faeces was still small. In another trial they treated pasture by scattering conidia mixed with a substrate of worm-free sheep faeces at intervals through the season, and claimed this reduced the counts of *H. contortus* and *O. circumcincta* larvae arriving on the pature from infected sheep. When scattered only once at the start of the season the method was only moderately successful on one or two occasions.

Grüner *et al.* (1985) fed sheep with *Dactylaria candida*, *Candelabrella musiformis* or *Arthrobotrys tortor* grown on bird seed. *D. candida* was recovered in 11% of 120 cultures of faecal material, *C. musiformis* in 10% and *A. tortor* in 43%. The number of cultures from which fungi were recovered diminished over three days, and the number of nematode larvae arising from faecal cultures increased. The quantities of fungus preparation fed to these sheep were large, 100g/6kg liveweight, which means that a 24kg sheep would have received 400g mouldy birdseed mixed with its 300g concentrate allowance. It is not surprising that the time taken to consume this was 3 to 6 hours for individual lambs.

A team of Danish workers made a major contribution towards finding a practical method for using fungi to control *Ostertagia* and *Cooperia* species in grazing calves. Nansen *et al* (1985) found pre-parasitic larvae of *Cooperia oncophora* were similar to free-living soil nematodes in their ability to stimulate trap formation in *A. oligospora* . Grønvold *et al.* (1985) found third stage larvae of *Cooperia* spp. were susceptible to the traps of *A. oligospora,* with 100% killed in three days on agar plates and up to 99% killed in faecal cultures when conidia were added to cattle faeces at the rate of 2,500/g. At dose rates of 8 and 25 conidia/g, yields of third-stage larvae were higher than the controls, but at 250 conidia/g, a 70% reduction was achieved.

Grønvold *et al.* (1987) conducted a series of experiments with *A. oligospora* incorporated into the faeces of cattle infected with *Cooperia oncophora.* Sporulating mycelium (10g) was mixed with 1kg faeces, and this was placed on pasture as a cow pat. A similar pat was made without fungus, and the concentrations of infective larvae in the pats and on surrounding grass monitored over the next 70 days. The fungus reduced both peak levels by more than 90%. In a second experiment, the entire contents of petri dish culture of the same fungus were milled and added to faeces at the rate of 150g/kg. From the ten replicates in this trial there was a mean peak reduction of 86% in pasture contamination, attributed to the fungus.

Nansen *et al.* (1988) investigated the ability of animal-parasitic nematode larvae to induce trap formation in *A. oligospora.* The cattle parasites, *Cooperia oncophora, Ostertagia ostertagi,* the sheep parasites, *Cooperia curticei* and *Haemonchus contortus,* and the equine cyathostomes, were equal to soil nematodes in stimulating trap formation. All these nematodes were better than the pig parasites, *Oesophagostomum dentatum* and *Oe. quadrispinulatum* and the mouse parasite, *Nematospiroides dubius,* at inducing trap formation. The cattle lungworm, *Dictyocaulus viviparus*, was a poor trap inducer, but was readily captured when traps were pre-induced. All species were equally susceptible to capture. The differences in trap-inducing ability

were correlated to differences in locomotive activity between the nematode species.

Grønvold *et al.* (1988) inoculated cow pats with 2,000 conidia of *A. oligospora* per gram, to reduce levels of *O. ostertagia* in faeces and on surrounding herbage. In an extension of this work, Grønvold *et al.* (1989) introduced grazing calves to the fields on which artificial cowpats had been placed, and found calves from the treated paddock developed worm burdens 37% lower than the controls, even though the reduction in pasture contamination after turnout was 71%. This time mycelial fragments of fungus were prepared from large scale liquid culture material, and added to faeces at the rate of 0.250 g/kg faeces. This inoculum resulted in reductions of 42% and 50-71% in larval counts from faeces and herbage respectively.

To overcome the difficulties experienced in achieving survival of spores during passage through ruminants, Larsen (Thesis, 1991) considered the use of fungi other than *A. oligospora* in controlling parasitic nematodes. He developed an *in vitro* stress selection technique to screen fungi for the ability to survive passage. This was used (Larsen et al., 1991) to select six *Arthrobotrys* species and seven *Duddingtonia* species for further selection in dung pat bioassays. *Arthrobotrys* and *Duddingtonia* species reduced *Ostertagia ostertagi* third stage larvae by approximately 75% and 96% respectively. These isolates were later grown on barley grains and fed to ruminating calves (Larsen *et al.*, 1992) in a successful *in vivo* passage trial. Not only were fungi re-isolated from the calves faeces, but reductions of third-stage larvae of up to 99% were found in dung pat bioassays and faecal cultures prepared by mixing these faeces with faeces from infected calves. Larsen considered that the thick-walled chlamydospores produced by these fungi enabled them to survive passage through the digestive tract.

Larsen (*pers comm*) spent a year at the CSIRO McMaster Laboratories working with Peter Waller and Margaret Faedo towards developing biological control for worms in grazing sheep. They screened hundreds of faecal samples from cattle, sheep and

minor species searching for fungi that had the ability to survive passage through the ruminant digestive tract. Several strains of *Arthrobotrys* and *Duddingtonia* were isolated and tested for their activity against gastrointestinal trichostrongyles (Larsen *et al.* 1994).

In the initial screening studies (Waller and Faedo, 1993), 94 fungi from a European collection were tested for their ability to reduce populations of animal-parasitic infective larvae. Six *Arthrobotrys* species, two *Geniculifera* species and two *Monacrosporium* species were selected for further trials.

These trials (Waller *et al.* 1994) included both the *in vitro* method of Larsen *et al.* (1991) and *in vivo* trials in which fungal spore preparations were administered to sheep both orally and via abomasal catheter. Samples were collected from the abomasum, the ileum and the faeces at time intervals after dosing. Samples were incubated in vermiculite culture, enriched with bait nematodes, for two weeks at 25°C and examined for nematophagous fungi. The authors concluded that conidia of certain fungi can survive gut passage unprotected, as fungus was isolated from each sampling point on at least one occasion.

As part of the screening studies, sheep faeces with 100 to 22,000 eggs/g faeces were mixed with conidia at the rate of 250/g to investigate the effect of prey density on predacity of the fungus. No effect was found, with capture rates greater than 80% at all prey densities. The quantity of faeces cultured (5-50g) also had no effect on the efficiency of the fungi. Two fungal species consistently performed better than the others tested. These were *A. oviformis* and *G. eudermata* . Both fungi reduced *H. contortus* larvae by over 90% in faecal cultures containing 50 conidia/g.

CHAPTER 4

PROGRAM OF INVESTIGATIONS

4.1. Introduction

The investigations conducted were directed at four areas:
1.- the possibility that trapping fungi have become associated with sheep grazing and already play a role in the ecology of parasitism.
2.- the ability of *Arthrobotrys oligospora* to withstand passage through the digestive tract of sheep and cattle.
3.- the potential for a trapping fungus to reduce the survival of pre-parasitic nematodes in whole sheep faeces in a pasture environment.
4.- the methods, dose rates and frequency of application of trapping fungi required for effective reduction of pasture infectivity.

The answers to the first question came from two investigations. The first was a survey of some pasture soils from Tasmanian sheep grazing properties, for trapping fungi. This provided some data on the occurrence of these organisms in the pasture environment. The other information came from glasshouse trials in which the effect of soil-borne trapping fungus on pasture infectivity was examined.

The second item was investigated *in vitro*, using the Rusitec (Czerkawski & Breckenridge, 1977) artificial rumen device to determine the ability of spores to survive in the rumen, and *in vivo*, using housed sheep, to investigate the passage of viable fungus in their faeces after administering preparations of *Arthrobotrys oligospora*.

The third item was investigated in a glasshouse using square plastic tubs of ryegrass and clover grown in potting soil. Contaminated faeces, collected from parasitised sheep, were placed on the soil and subjected to various fungal treatments.

Grass was cut at suitable time intervals, and nematodes recovered from washings of the clippings were counted.

The fourth investigation also used pasture grown in tubs in a glasshouse. The method of application adopted in the light of previous investigations was to spray the fungus directly onto contaminated faeces lying on the pasture sward.

4.2. Summary of Experimental Program

A complete description of materials and methods used in each investigation has been included with the results in the following chapters. Common procedures are described below.
Investigations are described under the following headings:

1. Farm Soil Survey
2. Investigations into Nematode/Fungus Interactions
3. Fungus Formulation Tests
4. Survival of Fungus During Passage through Ruminants
- *In Vitro* Experiments in the Rusitec
- *In Vivo* Experiments
5. Glasshouse Trials
- Effect of inoculum in soil or sprayed onto faeces
- Spray dose rate trials
- Timing trial

4.3. Materials and Methods

Procedures that were common to several investigations are described below.

Baited Plates

The baited soil plate method described by Barron (1977) was used to detect the presence of trapping fungi in faeces from experimental animals, and to detect nematophagous fungi in soil samples.

Between 0.5g and 1.5g of soil or faeces was sprinkled over a sterile petri dish containing 2% water agar. In the case of dry crumbly material, sprinkling usually resulted in a sparse covering of crumbs or particles, with large areas of clear agar

surface between them. When material was moist, it was spread in a star pattern, so that large areas of agar remained clear around the arms of the star. This was important to ensure that trap-bearing hyphae could be detected when they emerged from the sample material.

Plates were baited by placing two drops of a dense suspension of nematodes on a clear agar space. Plates were incubated at 23°C and observations made at various times under a dissecting microscope.

Bait Nematodes

Nematode larvae were infective juvenile *Heterorhabditis bacteriophora*, supplied and grown commercially by Ecogen, Australia, 63 Chapel St, Glenorchy, Tas.. Infective larvae were taken from three week old monoxenic cultures grown at 23°C in 500 ml Erlenmeyer flasks. Larvae were collected from culture media in modified Baermann funnels and washed by sedimentation in tap water three times before use. These larvae were allowed to migrate through paper tissues to exclude adults before use on baited plates. Bait nematodes were usually harvested one day before their use. They were maintained in aerated tap water at 23°C and concentrated by sedimentation immediately before use. Each drop of a heavy suspension contained approximately 10,000 of these nematodes.

Stock Cultures

Fungi were maintained in the laboratory on Oxoid potato dextrose agar in 90 mm petri dishes. Rapid production of plate cultures for use as inoculum in larger scale grain cultures was facilitated by inoculating sterile media in the following way. A sporulating mature plate culture was inverted over the open sterile plate and given a sharp tap on its base, causing a shower of conidia to fall onto the fresh medium. Plates inoculated in this way were ready for use after four days.

CHAPTER 5

SURVEY OF GRAZING PROPERTIES

5.1. Introduction

Survey

The nematode-trapping fungi are considered to be ubiquitous (Barron 1977) and may already form part of the ecosystem in which developing larvae of parasitic nematodes are exposed. If certain fungi have become associated with grazing sheep, this could indicate the existence of a predator/prey relationship between the parasites and a fungus. If, however, no such relationship exists, then the effect of introducing the predator to the ecosystem should be investigated. If the need arose, later in the project, to conduct trials on ground that was free of nematode-trapping fungi, to assess the effect of their introduction, knowledge of their distribution would be needed. Soils from a number of Tasmanian sheep grazing properties were sampled for the presence of nematode-trapping fungi. The fungi isolated were compared to the other local strains of nematophagous fungi for selection of the best candidate to use in the intended glasshouse trials.

Selection of Trapping Fungus

Fungi isolated from farm soils were compared with a previously isolated *Arthrobotrys oligospora* for their growth rate on laboratory media and their predacity against bait nematodes. The intention was that the most predaceous fungus available would be used in other trials.

5.2. Materials and Methods

5.2.1. Selection of Sampling Sites

Sampling sites were selected in consultation with Warren Barr and John Bushing of the Department of Primary Industry and Fisheries, on the basis of infection data gathered over 14 months from "Weaner Watch", a sheep parasite control program. This program monitored groups of lambs at monthly intervals over a year, and recorded drenching data and movements to different paddocks as well as liveweight and faecal egg counts. Comparing data from different farms indicated that, in some cases, lambs were continuously reinfected, acquiring worm burdens repeatedly in spite of frequent drenching. In other cases, faecal

egg counts remained low without the use of anthelmintics. Paddocks to be sampled were selected from the properties on the program, to find out whether the occurrence of trapping fungi correlated with the severity of parasitism in the sheep grazing there. The geographical locations of the properties visited are shown in fig. 5.1.1. The photographs on the following pages illustrate the diversity of sites sampled, which included lush, improved pastures; dry, treeless, unimproved grasslands; lightly timbered hillsides; some recently cleared, marginal grazing land; and some long-established, permanent pasture.

Sampling was carried out on two occasions: the first on the 28/10/91 when Warren Barr provided four wheel drive transport over large areas near Ross, in the Midlands, and the second on the 20/11/91 and 21/11/91, when Warren Barr provided transport over Northern Midlands properties and John Bushing provided access to Fingal Valley and East Coast properties. It would have been more informative if sampling had been repeated at different times of the year to assess the effects of season and rainfall on the occurrence of nematophagous fungi. The difficulties involved in arranging transport and access to properties, together with the extra laboratory work required to assess samples, could not be met with the limited resources available to the project. During the months of October and November, however, faecal egg counts tend to rise rapidly in Tasmanian lambs.

5.2.2. Sampling method

Sites were sampled by cutting small pieces from the top two or three centimetres of soil with a pocket knife from random points within the area to be sampled, until a 250ml takeaway food container was filled. Where large areas were sampled, soil was collected from random points along a zig-zag path across the area. Filled containers were sealed, labelled, and placed in an insulated container for transport to the laboratory. Where paddocks contained obvious sheep "campsites", usually heavily contaminated with manure, these sites were sampled separately. Otherwise, there was no attempt to deliberately include or avoid manure in samples.

5.2.3. Isolation of Fungi from Soil

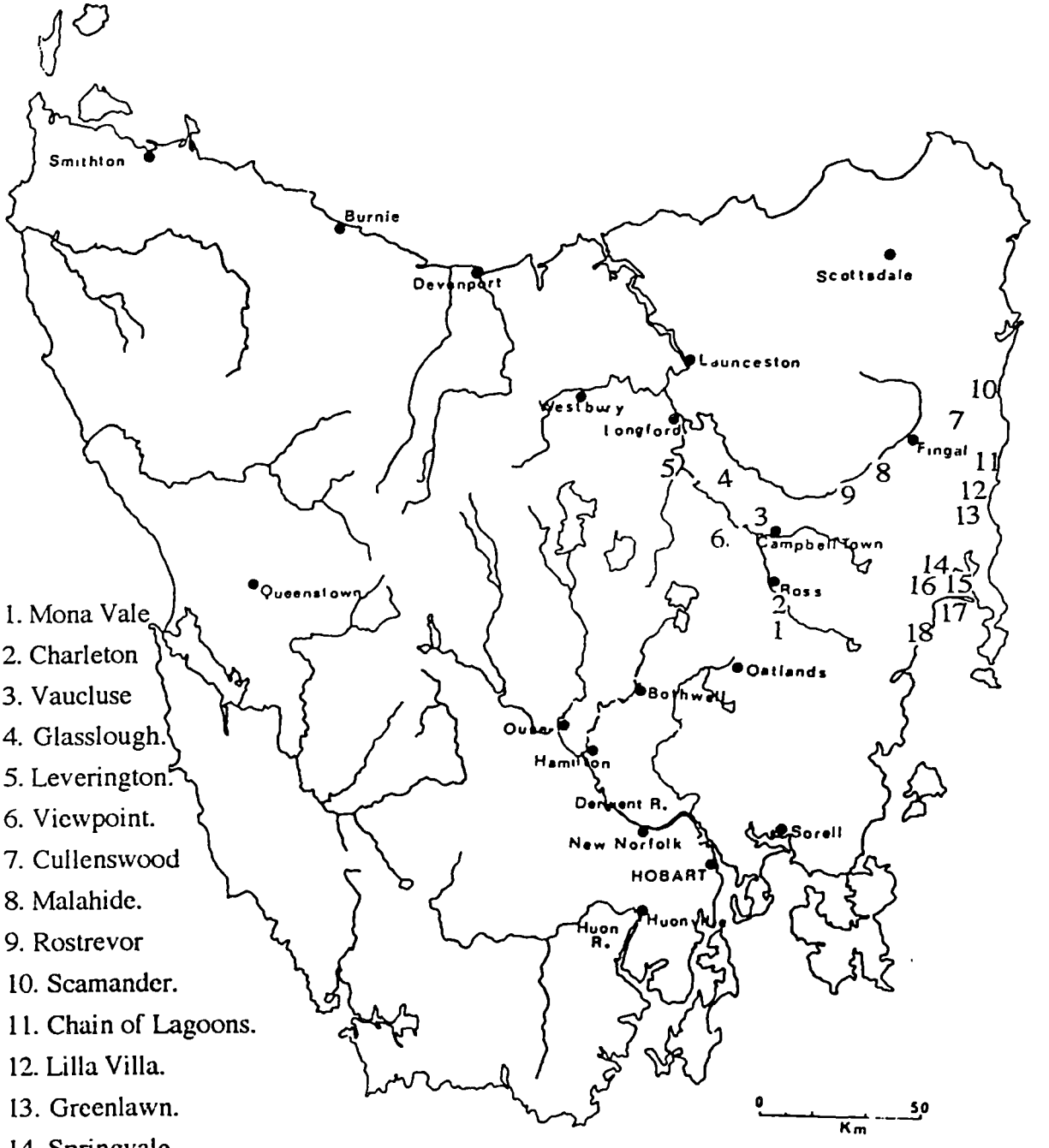
In the laboratory, soil samples were mixed within the containers and clumps of grass roots and other large objects such as stones were removed. Six baited plates were prepared from each sample, using 0.5-1.5g of soil sprinkled over 2% water agar in a 90mm petri dish. Two or three drops of a heavy suspension of infective juvenile *Heterorhabditis bacteriophora* were added, and the plates were incubated on the laboratory bench at ambient temperatures. Plates were searched for signs of nematophagous fungi under a dissecting microscope at intervals over the following seven or eight weeks.

When trapping fungi were observed, plates were put aside until the fungi sporulated. Conidia were picked off with a sterile needle and transferred to plates of sterile potato dextrose agar. Those which grew successfully were transferred to PDA slopes for storage, and identified using the key of Cooke and Godfrey (1964).

5.3. Results

The areas sampled included a variety of soil types and ranged from recently cleared land to well established pastures, including some on which sheep have been grazed for over a century. Samples were also taken from recently cultivated soils under crops of barley, rape and poppies. Many of the paddocks sampled were very large, some were open forest, and although the sampling technique used was intended to provide a representative sample of the whole area, it is possible that some patches of trapping fungi were missed. However, the sampling technique did gather soil from random points over large stretches of grazing land, and the results obtained should give a fair indication of the frequency of occurrence of predatory fungi. A description of sampling sites included in the survey appears in the appendix. A summary of the results appears in table 5.3.1.

Fig. 5.1.1. Map of Tasmania showing the location of properties sampled.



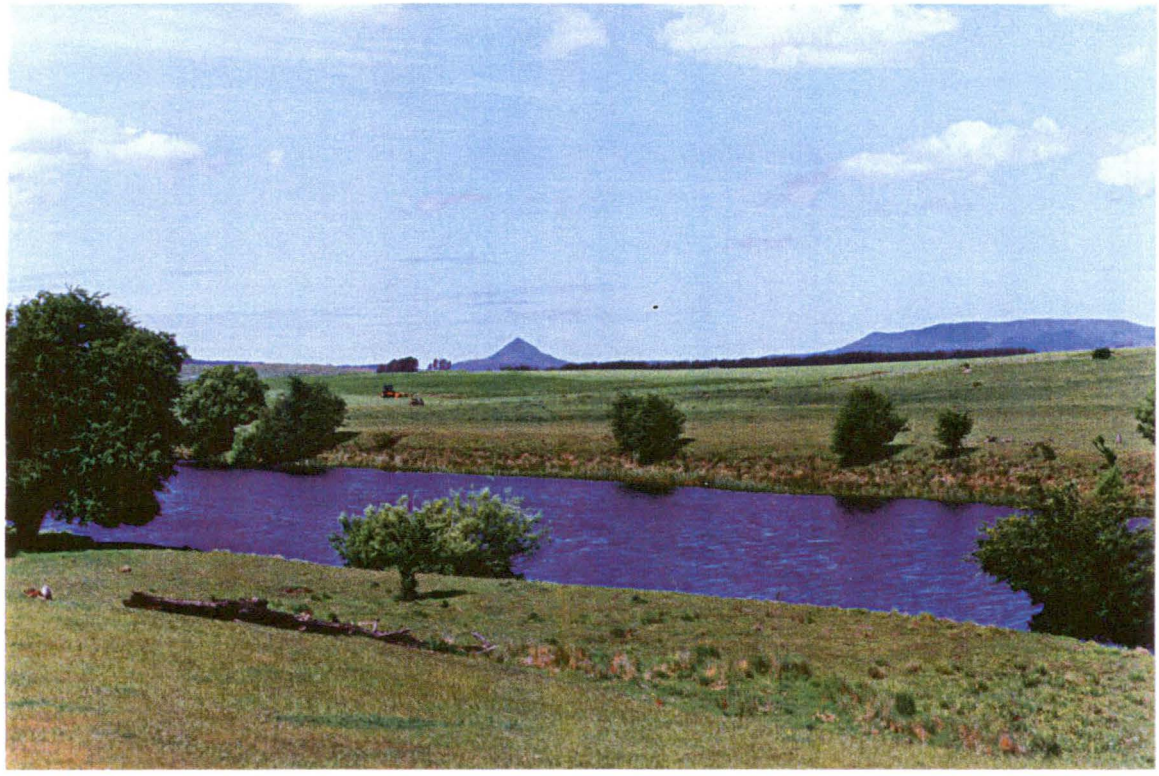
1. Mona Vale.
2. Charleton
3. Vaucluse
4. Glasslough.
5. Leverington.
6. Viewpoint.
7. Cullenswood
8. Malahide.
9. Rostrevor
10. Scamander.
11. Chain of Lagoons.
12. Lilla Villa.
13. Greenlawn.
14. Springvale.
15. Dolphin Sands.
16. Belmont.
17. Swansea Beach.
18. Kelvedon.

Pasture and open bushland at Charleton, near Ross

Chapmans Marsh paddock at Mona Vale

"The Marshes" at Glasslough, Epping Forest

Vaucluse, view from the "Ram Paddock"



Malahide, on the banks of the South Esk, Fingal

Paddock at Rostrevor, near Avoca

Old farmhouse at Kelvedon, Swansea

Recently cleared land at Lilla Villa, near Bicheno

Unimproved pasture at Kelvedon

Cullenswood, near St Marys

Table 5.3.1.

Summary of Survey Results

Properties Surveyed	**18**
Sites Sampled	**98**
Sites with Trapping Fungi	**28**
Properties with *Arthrobotrys*	**5**
Properties with *Dactylella*	**6**
Properties with others	**3**
Number of *Arthrobotrys* strains	**5**
Number of *Dactylella* strains	**6**

From a total of 98 soil samples, 28 produced nematode-trapping fungi. Of 18 properties visited, no trapping fungi were isolated from six and two others had unidentified slow-growing species that were very localised (for example, one found in swampy soil at Glasslough). *Arthrobotrys* was found in samples from five properties including four from recently cultivated soils and one from sheep yards. *Dactylella* was found on six properties and tended to occur at more than one sampling site, the majority of which were under well established pastures.

Detection of Nematophagous Fungi

Trapping fungus was observed in some plates after ten days, but most positive plates were detected up to five weeks after baiting. Observations were continued for seven weeks but the appearance of mites in some soil plates during this time raised the possibility of phoretic transport of conidia between petri dishes and observations were terminated at this time.

Fungi were identified as belonging to the genera *Arthrobotrys* or *Dactylella* by their conidia and conidiophores. Other types were observed in a few cases and appear under the heading "Others". The most common species of *Dactylella* formed adhesive networks, and bore 4-septate conidia singly at the conidiophore apex. These were identified as *Dactylella megalospora*. All the *Arthrobotrys* species formed adhesive networks, and were similar to *A. oligospora* , bearing whorls of conidia on simple

conidiophores. However, the conidiophore structures varied on different media, and the identification of some of these species may have been wrong.

Among the "other" species were knob-formers from Cullenswood, Lilla Villa and Rostrevor, identified as strains of *Dactylella lysipaga,* and a constricting ring-former from Springvale, identified as *Dactylella brochopaga.* The isolate, O3, from Greenlawn, was lost before it was identified.

Arthrobotrys strains and sites where they were found:

A3 Cullenswood, St Marys, under rape crop.
A2 Rostrevor, Avoca, in gravelly sand under oat crop and in finer grey soil south of highway.
A6 Scamander, in sandy loam under permanent pasture.
A4 Springvale, Cranbrook, in chocolate loam under a poppy crop and in red-brown soil under pasture.
A5 Lilla Villa, in the yards beside the shearing shed.

Dactylella strains and sites where they were found:

D1 Charleton, Ross, at sites 2, 5, 7 and 8 in Anstey's Hill Run and in the Cattle Paddock at sites 2 and 4, all under pasture.
D2 Kelvedon, Swansea, under pastures around old house and in adjascent paddock.
D3 Leverington, Epping Forest, in the black soil side of the Strawberry paddock and on river flats.
D4 Rostrevor, Avoca, in grey soil south of highway
D5 Malahide, Fingal, at sites 1, 3, 7, 8, 9 and 10, all under pasture and under a peppermint crop at site 6.
D6 Viewpoint, Isis, in the Slip paddock.

Sites where other types found:

O3 Greenlawn, Bicheno, in grey soil.
B52 Glasslough, Epping Forest, in swampy ground under reeds in the "Marshes".
M43 Springvale, Cranbrook, in "iron-stone" soil.
E31 Cullenswood, St Marys, in brown sandy soil on plain.
J13 Lilla Villa, grey soil under pasture.

5.4. Selection of Trapping Fungus

Fungi isolated from soil samples were cultured on potato dextrose agar at 23°C. Growth rates were determined by measuring colony diameter after incubation on both corn meal agar and potato dextrose agar for nine days.

Trapping behaviour was observed by placing agar blocks cut from the growing edges of plate cultures on water agar plates, which were then baited with infective juvenile *Heterorhabditis bacteriophora*.

5.4.1. Comparative Growth Rates of Isolates

Colony diameters of isolated fungi grown on PDA for six days appear in table 5.4.1.

Table 5.4.1. Comparison of Growth Rates

Colony diameter (mm) was measured after incubation of a single conidium on potato dextrose agar for 4 and 6 days.

Fungus	4 Days	6 Days
D5	30	37
D2	22	28
D4	9	12
D1	18	23
D6	9	12
A1	36	45
A3	34	46
A6	52	60
A4	48	58
A5	25	30
A2	60	68
O3	17	20
B52	5	6
M43	6	9
J13	6	10
E31	15	17

Generally, the fastest growing strains were *Arthrobotrys*, which, with the exception of A5, grew faster than the *Dactylella* strains.

The fastest growing *Dactylella* strain came from Malahide, and the fastest *Arthrobotrys* came from Rostrevor. Other genera grew very slowly. The laboratory strain of *A. oligospora* (A1), grew less rapidly than some of the other fungi tested.

5.4.2. Comparison of Trapping Activity

Blocks of agar cut from second growth rate trial plates were used for comparison of trapping activity. Where no growth occurred the strain had been lost and could not be included in the list below. The distance between the edge of the spreading hyphae and the agar block is given, and the number of "+"s indicates the quantity of traps formed. Where traps other than networks were formed, the type is given.

Table 5.4.2. Growth and Trapping Activity of Isolates.
Observation times are given in hours and days.
Numbers are the extension growth in mm.
Where traps other than adhesive networks grew, the type is given, eg knobs, where traps were sticky knobs, con. rings, where traps were constricting rings.
Trap appearance is denoted by +.
The number of +s indicates the intensity of trapping.

Fungus	24Hr	32Hr	48Hr	72Hr	5Days
J13	1	1	2	4+ knobs	5+
M43	-	-	-	2+ con. rings	
E31	-	-	-	3	6+ knobs
D2	3+	7+	10+	13++	20++
D4	2	2	4	5+ knobs	10+
D5	3	5+	8++	13++++	20++++
A1	5+++	7++++	12++++	17+++++	25+++++
A2	2	6	12	17++	40++
A3	5+	8+	12+	17+	40+
A4	5+	8+	12+	16++	40++
A5	2	4	7+	12+++	20+++
A6	5+	8+	11+	17+++	25+++

Trapping behaviour varied considerably among the *Arthrobotrys* strains. In A6 and A2 traps were formed in a ring around the agar block with few traps within 10mm of the block and few traps in the outer 10mm spread of the hyphae. In contrast to this, A5 formed traps from the agar block to halfway along the spread of the hyphae. In A2 and A4, hyphae covered the whole plate but traps were sparse and patchy. A1 and D5 developed numerous dense bundles of adhesive networks spread over all colonised parts of the plate, and trapped a lot more nematodes than the others.

The knob- and constricting ring-formers caught very few nematodes and were very slow-growing. These attributes would make them difficult to mass-produce and unlikely to be of any use in controlling pre-parasitic gastro-intestinal strongyles.

The fungus, D5, identified as *Dactylella ellipsospora* was relatively predaceous but sporulated sparsely compared to *A. oligospora*. Conidia were borne singly on unbranched conidiophores. Spore yields would have been several orders of magnitude smaller from a plate of this fungus than from a plate of sporulating *A. oligospora*. This attribute precluded D5 from further testing; its slower growth rate and low spore yield would have made mass production difficult.

The previously isolated strain of *A. oligospora*, A1, was relatively rapid-growing compared to most fungi tested and was much more predaceous. It began vigorous trapping two days earlier than the next best predator, D5, and was the most successful of all fungi tested at all observation times. The ease with which it could be mass-produced and its abundant sporulation were also considerations in its choice for use in further tests.

CHAPTER 6

FUNGUS / NEMATODE INTERACTIONS

Introduction

Assuming the fungus could be introduced to the pasture environment, its ability to control nematodes would depend on its predacity and the extent to which it colonised the habitat of the nematodes. The duration of trapping activity and the effect of prey density on the intensity of trapping could also be important to the control of pasture infectivity. For example, if the threshold nematode density for trap induction were too high, the fungus may be ineffective in controlling pasture infectivity until parasite numbers were already at an unacceptably high level. Also, if trapping effort declines while nematodes continue to arrive on the pasture, the fungus may be ineffective. The extent to which the fungal inoculum colonises the substrate could also affect the proportion of the nematode population removed by trapping. Moreover, if developing fungal colonies encounter nematodes, will the formation of traps limit further hyphal extension? The following two experiments were conducted to investigate these aspects of fungal behaviour.

6.1 Effects of Prey Density

The effects of fungal inoculum size and prey density on initial fungal establishment and on predacity were investigated.

Agar plates without nutrients were inoculated with fungal spores at low, medium or high density rates. Bait nematodes were later added at three levels of density, and the response of the fungus was monitored over several days. Additional nematodes were added at weekly intervals, and the activity of the fungus monitored.

Materials and Methods

Arthrobotrys oligospora conidia were collected from mature petri dish cultures in sterile distilled water.

Petri plates were prepared using 2% agar in tap water.
Fungal spores were added in aliquots of 0.1 ml sterile distilled water at the rates of 7, 70 or 700 conidia per plate, The spores were spread with a sterile glass rod. Plates were allowed to dry overnight at 23°C and germinated spores were counted under a dissecting microscope before nematodes were added.

Nematode larvae were collected after migration through paper tissues to ensure only live, infective juveniles were used. Quantities of 5,000, 500 or 50 larvae were added to the plates in aliquots of 0.5 ml water. Three replications of each nema density were made for each fungal spore density. After one week, it was clear that greater numbers of nemas were required as the larvae tended to crawl off the agar and up the sides of the dishes. Infective larvae were then added at the rate of 5,000 (low dose) or 50,000 (high dose) at weekly intervals.

Scoring - The fungus formed traps either in highly developed networks or in less complicated formations of less than five loops together. These were scored as HDT for high density trap bundles or LDT for low density traps. An estimate was made of the percentage of the plate surface occupied by traps at each weekly inspection.

Plates were incubated at 23°C and inspected daily for signs of trapping, sporulating and clearance of nematodes. Observations were continued for three weeks.

Results

Results are summarised in table 6.1.1.
After two days traps had appeared on all plates except for those dosed with only 50 nemas. By the end of one week, however, plates dosed with 500 nemas had been cleared of worms and in some cases trapping had ceased. The plates with the highest rate

of fungal inoculum had been cleared of nematodes in both 500 and 5,000 treatments, but had formed no traps where only 50 nemas had been added. Those inoculated with 70 conidia were still actively trapping if 5,000 nemas were added, two of the 500 rate plates had been cleared and the other had ceased trapping. Where only seven conidia were present, high density trapping had caught most of the 5,000 nemas added, but with only 500 nemas, trapping was continuing in one replicate, another had empty traps and in the third the traps formed earlier in the week had disappeared. It was this observation that led to the decision to use greater numbers of nematodes when re-baiting the plates.

Three days after re-baiting, all plates showed trapping activity. Those in which trapping had not previously been induced were all very active, but those which had been trapping but were unsuccessful in capturing nematodes and had subsequently ceased trapping were not very active. These were the plates which had received 500 nemas initially, with inocula of 7 or 70 conidia. Those inoculated with 700 conidia and baited with 500 nemas, resumed trapping quickly after re-baiting, and were almost cleared of nematodes by the end of the week.

Table 6.1.1. Effects of Varying Inoculum Size and Prey Density.
Water agar petri plates were inoculated with three different quantities of conidia, and baited with three different quantities of nematodes.
Plates were re-baited after weeks 1 and 2.
"Nemas remaining" were those on plates at the end of the third week.
The key to symbols used in the table appears below.

Fungus Rate	Baiting Rate	Time of Observations 2d	4d	7d	2 weeks	3 weeks	Re-Bait Rate	Nemas Remaining
7	50	N	N	N	10% HDT	30% LDT	Low	Many
7	50	N	N	N S	10% HDT	dried up	Low	-
7	50	N	N	N	10% HDT	10% HDT	High	Few
7	500	T	T	N	<5% LDT	10% LDT	Low	Many
7	500	T	T S	N	10% LDT	20% LDT	Low	Many
7	500	T	T	T	20% LDT	30% HDT	High	Many
7	5000	T	T	T	5% LDT	<5% LDT	Low	Many
7	5000	T	T	T	<5% LDT	<5% LDT	Low	Many
7	5000	T	T	T	20% HDT	30% HDT	High	Few
70	50	N	N	N	50% HDT	50% HDT	High	Few
70	50	N	N	N	30% HDT	30% HDT	High	Few
70	50	N	N	N	10% LDT	10% LDT	Low	Many
70	500	T	T	T C	30% HDT	30% HDT	High	None
70	500	T	T	N	<5% LDT	20% LDT	Low	Many
70	500	T	T	T C	40% HDT	30% HDT	High	None
70	5000	T	T	T	30% HDT	30% HDT	High	Few
70	5000	T	T	T	5% LDT	5% LDT	Low	Many
70	5000	T	T S	T	40% HDT	50% HDT	High	None
700	50	N	N S	N S	50% HDT	50% HDT	High	None
700	50	N	N	N	40% HDT	40% HDT	High	None
700	50	N	N	N S	10% HDT	10% HDT	Low	Many
700	500	T	T S	T S C	40% HDT	50% HDT	High	None
700	500	T	T	T S C	50% HDT	60% HDT	High	None
700	500	T	T S	T S C	40% LDT	20% LDT	Low	Many
700	5000	T	T	T S C	70% HDT	60% HDT	High	None
700	5000	T	T	T S C	50% HDT	50% HDT	High	None
700	5000	T	T	T C	70% LDT	50% LDT	Low	Many

Observations during first week:
T = Trapping, S = Sporulating, C = Cleared plate of nemas
N = Not Trapping

Later observations describing trapping intensity:
LDT = Low density trapping bundles (few loops)
HDT = High density trapping bundles (many loops)
Percentage figure is estimate of plate area occupied by traps.

Number of bait nematodes added at re-baiting:
Low = 5,000 nemas per plate ; High = 50,000 nemas per plate

Effects of Fungal Inoculum Size

There was a general trend throughout the experimental period for the higher fungal inoculum plates to have more of their surface occupied by traps than those which received less inoculum. Provided that nematode numbers were sufficient to induce trapping, greater numbers of nematodes were captured on these plates than on others.

At the lowest inoculum level, and when nematodes were plentiful, trapping during the first week almost cleared the plates of worms. After re-baiting, trapping activity declined, except where the higher dose of nematodes was added. In those plates which received only 50 nemas initially, both levels of re-baiting stimulated similar trapping activity, but in plates which had received 500 nematodes initially, re-baiting stimulated activity only at the higher rate. This effect was also evident at the medium inoculum rate, but was not noticeable at the top inoculum rate until the end of the third week. Those plates which had no traps during the first week performed better during the second week than others which had been trapping but were re-baited at the low rate.

Effect of Nematode Numbers

The addition of 50 nematodes failed to stimulate trapping in any plate. At the rate of 500 nematodes, trapping was induced but rapidly declined unless much larger quantities were added. The quantity of 5,000 nemas induced high trapping activity during the first week, but activity was not maintained unless greater numbers were added in subsequent weeks. At the end of the experimental period, those plates re-baited at the 50,000 rate were mostly cleared of live nematodes, while those re-baited with only 5,000 worms still contained large populations of live, active, nematodes.

6.2. Trapping vs Hyphal Growth

Observations of *A. oligospora* during trapping suggested there might be less extension growth of hyphae once trapping was induced. This experiment was designed to compare growth rates between trapping and saprophytic colonies. This comparison presented a certain amount of difficulty because growth would be affected by nutrition, and trapping cannot be induced on standard nutrient agar. As a compromise, tap water agar was used and nutrients provided in added broth. Plates receiving nematodes were given less broth, to compensate for the nutrients in the nematodes. Increase in colony diameter was used as a measure of growth.

Materials and Methods

Six water agar petri plates were inoculated with a single conidium of *A. oligospora*. After three days at 23°C colony diameters were measured.

Oxoid nutrient broth powder (0.5g) was dissolved in 50ml water. Three plates received two drops of this solution and the other three plates received one drop of nutrient broth and one drop of a heavy suspension of infective juvenile *Heterorhabditis bacteriophora*. Colony diameters were measured after a further three days' incubation. Where colonies had irregular shapes, the maximum diameter was measured.

Results

Results appear in table 6.2.1. One conidium failed to grow. After three days, colony diameters were variable and treatments were allocated to include large and small diameter colonies in the controls. Interestingly, when infective larvae came into contact with nutrient broth they exsheathed, but this did not prevent their subsequent capture in adhesive nets.

Table 6.2.1. Radial Growth of Predatory and Saprophytic Colonies
Colony diameters (mm) were measured before, and three days after the addition of nematodes or nutrients.

Initial Diameter	Phase	3 days later	Increase
15	Predatory	30	15
18	Predatory	40	22
10	Predatory	40	30
19	Saprophytic	70	51
12	Saprophytic	65	53

	Mean Increase
Predatory	22 mm
Saprophytic	52 mm

Extension growth rates of colonies in the predaceous phase were less than half those of saprophytic colonies.

Although the significance of this result is limited, due to the small number of replications and the uncertainty of the nutrient value of the nematodes compared to the broth, it was included in this report for two reasons. First, it is indicative of a reduction in the extension growth of colonies entering the predaceous phase. This could affect the numbers of viable spores needed to colonise a given volume of faeces if nematodes are present at germination. Second, the exsheathing of infective juvenile *Heterorhabditis heliothidis* when placed in nutrient broth was an unexpected phenomenon. The observation that they were nevertheless subject to predation was considered worthy of reporting.

CHAPTER 7

FUNGUS FORMULATION TESTS

Introduction

During the course of the project, several minor investigations were made into methods for harvesting fungal spores and preparing them for storage and delivery in sprays or pellets. The methods used and results obtained are reported together under headings for each investigation.

The production of fungal spores for use in experiments was achieved by culturing *A. oligospora* on sterile wheat grains. Mature grain cultures sporulated profusely, particularly when allowed to dry slowly during sporulation. The masses of conidiophores covering the grain were hydrophobic, and condensation from the container lids often lay in beads on top of them. This created difficulties in washing conidia from mature cultures, as matted conidiophores tended to pack together, trapping large numbers of conidia into impenetrable bundles. A method of spore harvesting was sought to overcome this difficulty.

Grain cultures of *Arthrobotrys oligospora* were dried and milled to produce spore powder for use in some early experiments. The spent grain residues were considered undesirable for inclusion in formulations because they could provide substrate for fungal competitors and antagonists, so a method of excluding spent grains was sought. A method for harvesting conidia from mature grain cultures was devised to be used in conjunction with clay/alginate pellet production. The survival of fungus in refrigerated liquid storage was monitored over several weeks. Another method for harvesting fungal spores using canola oil was tried, and the oil suspension was used in preliminary *in vivo* trials.

In order to escape rumen degradation fungal spores may require protection by encapsulation in a substance that will release them

further down the digestive tract. There have been a number of compounds devised for such a purpose (see Chapula 1975 for review). Formalin (Anon. 1971) has been used to protect proteins in feedstuffs from microbial attack in the rumen. Ferguson and Solomon (1966) found that a coating of casein treated with formalin protected methionine from degradation in the rumen. Two methods of preparing conidia in formalinised casein were tested.

Materials Methods and Results

7.1. Spore Powder Production and Assay

Quantities of fungus for use in trials were produced on whole wheat grains. A 50 ml beaker was used as a measure and a scoop for transferring wheat to 250 ml polypropylene takeaway food containers. A level beaker full of wheat was placed in each container, and an equal quantity of tap water added. Lids were then placed on the containers, and they were sterilised at 121°C in a pressure cooker for 40 mins. After allowing the cooked grain to cool, agar strips cut from four day old plate cultures were mixed into the grain, using a flamed spatula in a laminar flow cabinet. Containers were then incubated at 23°C for three weeks before use. Contaminated cultures could be detected by visual inspection without removing the lid of the tub. After three weeks, lids were removed to encourage the fungi to sporulate in response to drying.

Mature grain cultures were dried in a drying cabinet at 35°C with the lids removed from the tubs. Dried cultures were hammer-milled for the production of spore powder, and used intact when preparing liquid suspensions of spores for spray inoculum.

Assay procedure

Spore powder (1.00g) was stirred into 100 ml water. This suspension was used to make a dilution series up to 10^{-6}. Aliquots (0.1 ml) of each dilution were spread with a sterile glass rod on 3 sterile water agar plates. The number of fungal colonies arising was counted after two days' incubation at 23°C. Bait

nematodes were then added, and plates were examined for signs of trapping three days later.

The spore powder used in the glasshouse trials, the dairy cow and sheep passage trials, and the *in vitro* rumen survival trial, was estimated to contain 600,000 spores per gram.

7.2. Formalinised Casein

Method 1.

Equal quantities of spore powder and hydrolysed casein were mixed together and 5% formalin was added to form a stiff paste. This was then ground with excess casein powder to form crumbs, which were sieved out and dried at 35°C. Two muslin bags of crumbs were placed in a fermentation chamber of the Rusitec (Czerkawski & Breckenridge, 1979) rumen simulation device. One was removed after 3.5 hours and the other after 24 hours. After removal from the Rusitec, bags were washed in tap water twice before approximately 1 ml of the contents was spread on each of three baited agar plates. An untreated bag of crumbs was rinsed in tap water and plated as a control.

Results

All three baited plates sprinkled with casein crumbs which had been immersed in the Rusitec chamber for 3.5 hours produced trapping fungus, but none of the material which was immersed for 24 hours did. Thus, although the fungus survived the pelletising treatment, this failed to protect it from degradation during prolonged immersion in the Rusitec.

Method 2.

Casein (8g) was added gradually to 50ml 0.1N NaOH in a beaker on a magnetic stirrer, to make a 16% solution. This was adjusted to pH 9. Approximately 0.2g spore powder was stirred into 25ml of the solution before adding 0.8 ml formalin. As this mixture was stirred with a spatula, it crumbled into fragments. Some of these lumps of gel were placed directly onto 1/4 strength PDA plates and some onto baited water agar plates. The remainder was suspended in 200 ml water and administered to a sheep

from a glass bottle. Faeces were collected the following morning, and samples were spread onto seven baited agar plates.

Results

The fungus did not survive encapsulation by this method. None of the material produced by this method grew fungus on any media plates.

7.3. Clay/alginate Pellets

A modification of the method of Fravel *et al.* (1985) was used. Bentonite (50g) and 5g sodium alginate were mixed together dry and added gradually to 500ml water on a magnetic stirrer. Some of this was poured over a sporulating petri plate culture of *A. oligospora* and stirred to dislodge conidia. A syringe was used to transfer this slurry and dispense it dropwise, into a beaker containing 0.25M $CaCl_2$. Soft beads of gel were formed. These were dried at 35°C, and formed hard beads of about 2mm diameter. A portion of the wet pellets was administered to a sheep (see chapter 8).

Results

Fungal spores pelletised by this method remained viable and hyphae grew from pellets placed on nutrient media. Pellets dried at 35°C also produced hyphae readily. There was no viable fungus detected in the sheep faeces.

7.4. Spore Harvesting using Bentonite Slurry

When spores are washed from mature grain cultures, the hydrophobicity of the spores tends to prevent them from entering the water. The hydrophobicity problem is overcome by using a bentonite slurry, and the shear forces acting on conidia during mixing are greater in a thick slurry than in water, enhancing their removal from conidiophores. Addition of water after mixing reduces the slurry to a thin soup, which is easily poured off and readily passes through a strainer. This method allows spore harvesting from solid culture without including spent grain in the final formulation. For pelletisation, a solution of sodium alginate can be added. Provided the final alginate content

was between 1 and 2%, this mixture formed beads readily when dropped into 2% calcium chloride solution.

Bentonite was added to water in a kitchen blender running on "blend" until the suspension became too thick to flow in the blender. Some of this gel was placed in a plastic bag with mature fungus cultures and mixed by squeezing with the fingers. Water was added to thin the slurry, and grain was removed by passing the suspension through a strainer. This method was also used for harvesting spores from dried petri dish cultures. The number of viable spores per ml was determined by spreading 0.1ml aliquots of a dilution series on water agar plates and counting fungal colonies after 24 hours. Bait nematodes were then added to confirm that the colonies observed were nematophagous fungi.

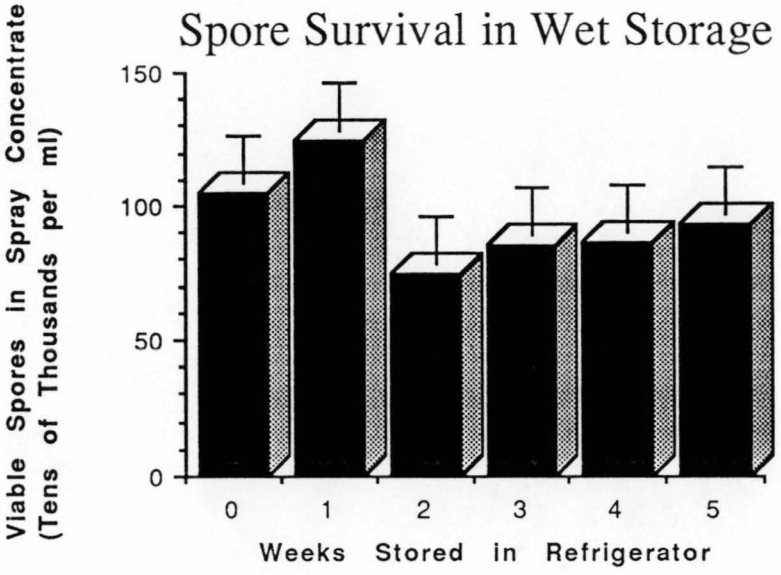
A suspension of spores thus obtained was stored in a refrigerator and tested weekly for five weeks.

Results

The results of assays are summarised in figure 7.4.1. Differences in the first two weeks results were probably due to sampling errors.
There was no significant death of spores over the storage period.

The fungal material for the third dose rate trial was collected in 500 ml bentonite slurry mixed with four tubs of grain culture and diluted to 800 ml. This was assayed at 450,000 spores/ml. Thus the yield from approximately 200 g of grain was 3,600 million conidia.

Fig. 7.4.1. Survival of Spores in Bentonite Slurry.
A slurry containing *A. oligospora* conidia was refrigerated. Samples were taken weekly and the number of viable propagules determined.



7.5. Spore Harvesting using Canola Oil

Canola oil was poured over mature petri dish cultures and stirred to displace conidia from conidiophores. The oil was poured off into a beaker which was covered with parafilm and left on the laboratory bench. After three weeks at room temperature, a droplet was placed on a water agar plate baited with nematodes. Plates were examined the following day for signs of trapping activity.

Results

Canola oil readily penetrated the conidiophores of mature fungal colonies, and conidia readily detached, forming a dense suspension in the oil. The test for viability after three weeks at room temperature produced an interesting result. The presence of nematodes induced conidial trap formation in these oil-borne spores. The day following inoculation with canola oil spore suspension, some nematodes could be seen moving about the plate with a conidium on a trap fastened to their necks, while other worms were struggling in firmly anchored traps. No estimate was made of the proportion of spores germinating, but very few ungerminated spores could be found.

CHAPTER 8

SURVIVAL OF FUNGUS DURING PASSAGE THROUGH RUMINANTS

Introduction

One key to the successful use of nematophagous fungi as biological control agents against animal-parasitic nematodes could be the ability of propagules to survive passage through the digestive tract of ruminants. This would enable the fungus to be administered orally, as a feed additive, a lick or a drench. The treated animals would then disperse the fungus in their droppings, which would supply a rich substrate for fungal growth and help establish the organism in the pasture environment. Placement of propagules within the faeces would also be ideal for reducing the survival rate of nematode larvae in their free-living phase. Ideally, spores from introduced fungal colonies would be ingested by grazing livestock, through which they would pass to perpetuate the presence of the control agent in the animals' faeces.

Although there are reports of nematode-trapping fungi surviving passage through ruminants (Gruner *et al.* 1985, Hashmi and Connan 1989), there has been no attempt to assess their survival rate. The strain of *Arthrobotrys oligospora* used in the current investigations showed good characteristics of growth rate and predacity, but its ability to survive passage through sheep and cattle was unknown. The following experiments were conducted to determine whether the fungus survived, and if not, where it was being killed.

Investigations were conducted *in vitro*, using a rumen simulation device, and *in vivo*, using housed sheep and pasture-fed dairy cows.

8.1. *IN VITRO* INVESTIGATIONS

Introduction

If fungal spores are to survive passage through the digestive tract of a ruminant animal, they must be capable of surviving for an extended period in the rumen. To determine their survival time, fungal spores must be subjected to the environment of a working rumen. While it is possible to surgically prepare a sheep with a rumen fistula (Hecker 1969), a laboratory-based *in vitro* rumen simulation device is a useful tool for preliminary investigations into these matters. The "Rusitec" was developed at the Hannah Research Institute (Czerkawski & Breckenridge, 1977, 1979) for nutritional investigations, and provides a similar physico-chemical and microbial environment to that found within a real rumen. This device was used to measure the survival of *Arthrobotrys oligospora* spores, and to test the ability of various preparations to protect them from degradation.

Materials, Methods and Results

The Rusitec apparatus was set up as described by Czerkawski and Breckenridge (1977), using inoculum from a sheep maintained on a diet of lucerne chaff and sheep pellets.

Bags containing approximately 10g lucerne chaff and sheep feed pellets (Gibsons Pty Ltd, Hobart) were placed in the fermentation chambers daily, with each bag remaining in the vessel for two days. Spent bags were squeezed by hand and the resulting fluid returned to the fermentation vessel.

8.1.1. Survival Time of Fungus in the Rumen

Bags containing 3g fungus spore powder were placed in each of the four vessels. At intervals of 1, 2, 3, 4, 24, 36 and 48 hours, a bag was removed, and a sub-sample of its contents spread on two baited, water agar petri plates. These plates were observed for signs of trapping fungi over the following week. A bag of spore powder was soaked in artificial saliva (McDougall 1948) briefly before plating as a control.

Results

All replicate plates spread with spore powder held in the Rusitec fermentation chamber for up to four hours developed trapping fungus, but the incubation period before fungus emerged increased with increasing immersion times. Samples immersed for one or two hours produced mycelium within three days. Three hours' immersion extended this to five days and four hours immersion caused very sparse growth which was only detected after seven days incubation. There was no growth from spores immersed for 24 hours or more.

After four days, trapping activity could be detected on the control plates, both 1hr plates and one of the 2hr plates. The other 2hr plate was doubtful, one of the 3hr plates was similar, and both 4hr plates contained contaminants, and the bait nematodes were moribund. No trapping fungus appeared on any of the other plates. At the end of the week, the distinctive conidiophores of *A. oligospora* could be found on the controls and the 1,2,3 and 4hr plates in progressively reducing quantities. No trapping fungus grew from material that had been in the rusitec vessels for longer than four hours.

8.1.2. Inhibition of Fungus by Rumen Contents

Sterile, potato dextrose agar petri plates were sown with a lawn of fungal spores. Solid material, from bags taken from fermentation chambers of the Rusitec after two days' immersion, was placed on one side of six plates, and a narrow strip of agar removed from three plates adjacent to the solid material. Another plate was prepared with wells cut into the agar, and these were filled with fluid from the fermentation vessels. Plates were incubated at 23°C for 24 hours and examined for inhibition zones.

No fungus grew within 10mm of the solid material but the spread of the inhibiting agent was prevented by removing a strip of agar. No inhibition was observed around the wells containing rumen fluid.

8.1.3. Survival of *A. oligospora* Conidia at Abomasal pH

If conidia could survive passage through the rumen, or could be protected by a substance which dissolved to release them in the abomasum, would they survive in the low pH (2.3-2.4 according to Comline and Titchen, 1961) typical of the abomasum? Conidia were held at pH 2.2 at 38°C for up to six hours, and their viability tested.

A citric acid buffer (Perrin & Dempsey, 1974) at pH 2.2 and another at pH 6.0 were prepared and sterilised. *A. oligospora* conidia were collected from a mature plate culture by swirling sterile water and glass beads. Aliquots of 1.0 ml of the suspension were placed in each of three test tubes containing 10ml buffer at pH 2.2, and held in a water bath at 38°C. A small volume was withdrawn from the bottom of the tubes with a sterile pipette and transferred to 10ml sterile buffer at pH 6.0 after 35 min., 140 min. and 6 hours. After allowing spores to settle to the bottom of the test tubes, three 0.1ml aliquots were withdrawn and spread on PDA plates which were incubated at 25°C for three days.

Result

After three days' incubation, trapping fungi were growing on all baited plates. Conidia of *A. oligospora* were able to survive the temperature and pH of the abomasum for at least six hours.

8.2. *IN VIVO* TRIALS

The investigations into survival of fungus after passage through animals were in three parts. A single sheep was used to test survival of the fungus given orally in various forms. When preliminary tests gave a positive result, a full-scale trial using four sheep was devised to test the proposed hypothesis. Cattle were also used to determine the ability of the fungus to survive passage. The baited plate method was used to detect the presence of viable fungus in faecal samples. A sensitivity analysis of this method was conducted before adopting it for use in the trials.

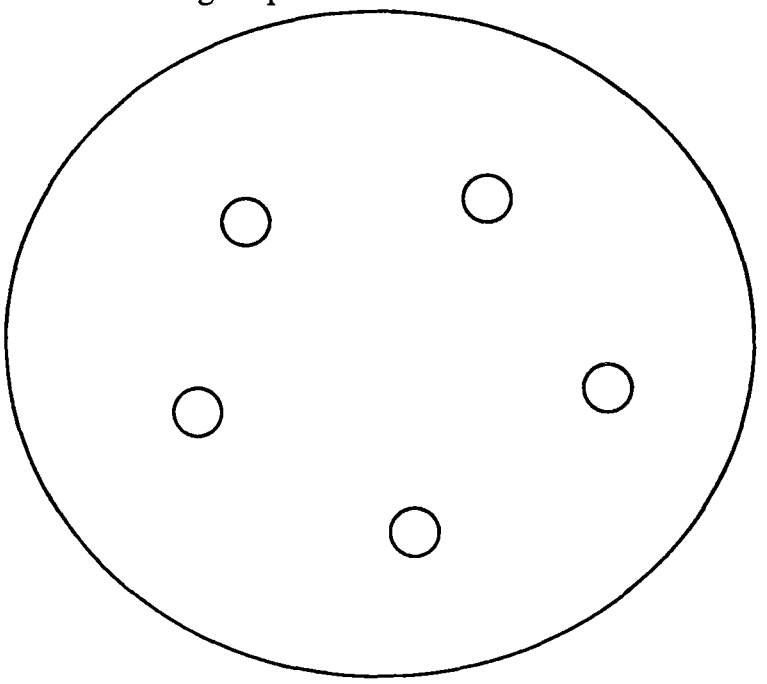
Merino wethers were used in trials to test the survival of the fungus during passage through sheep. These animals were individually penned indoors and fed on lucerne chaff and sheep pellets. Faeces were collected in calico bags attached to a harness on the animal. Faecal samples were spread on baited agar plates to detect viable fungus propagules.

Materials, Methods and Results

8.2.1. Baited Plate Sensitivity Test

Sheep faeces were crumbled and placed on 2% water agar in petri plates as shown. Small mounds of crumbled faeces about 3mm diameter and 1mm deep were placed approximately in a circle on the agar surface. Individual conidia of *Arthrobotrys oligospora* were drawn into a syringe needle from an aqueous suspension, under observation through a dissecting microscope, and then deposited onto one of the faecal mounds. In this way, the desired number of conidia could be added to each faecal heap.

Fig. 8.2.1. Baited Plate Sensitivity Test
The circles represent mounds of crumbled faeces, showing their pattern of placement on agar plates.



Each mound was inoculated with either 1, 2 , 5 or 10 conidia, with each plate containing five replications of one inoculum level, and two plates set up for each inoculum level. A single drop of a dense suspension of infective juvenile *Heterorhabditis bacteriophora* was placed in the centre of each dish, and the plates were incubated at 23°C.

After three days the plates were examined daily for the presence of traps around each faecal mound. Each plate received a score out of a possible five for the number of mounds from which trapping fungus had emerged.

Results

After three days, no traps had emerged but the bait nematodes were moribund. Plates were baited again on day four and observations continued. The next day, traps were visible close to some of the faecal mounds. More trapping hyphae emerged during the following days until observations were discontinued after eleven days. The complete results appear in table 8.2.1.

Table 8.2.1. Results of Baited Plate Sensitivity Test

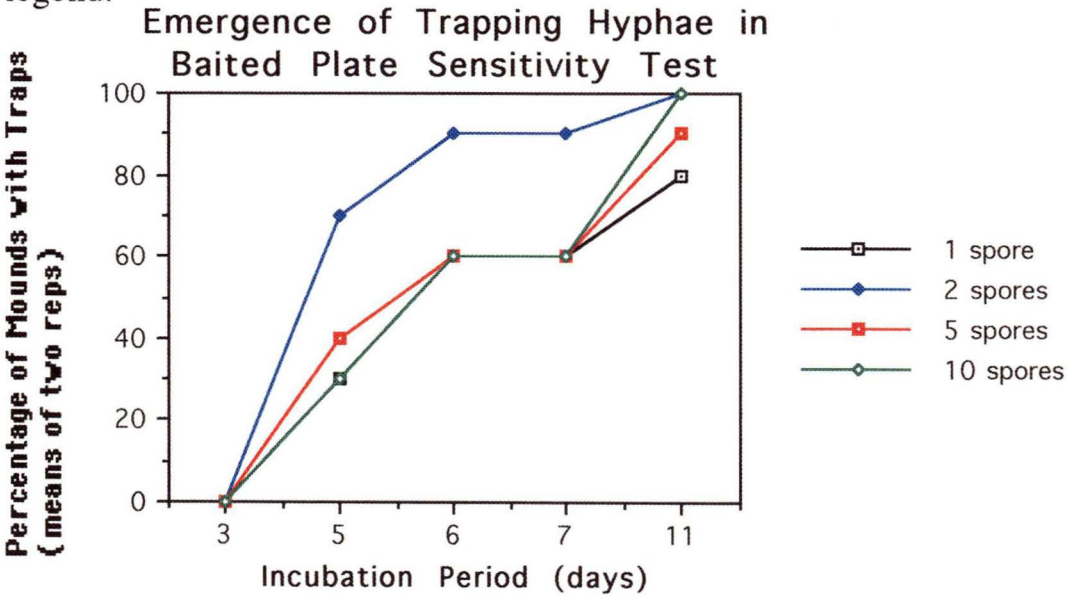
Number of faecal mounds growing trapping fungus.

Number of	Times of Observations				
Conidia	day 3	day 5	day 6	day 7	day 11
1	0	2	4	4	5
1	0	1	2	2	3
2	0	4	5	5	5
2	0	3	4	4	5
5	0	3	4	4	5
5	0	1	2	2	4
10	0	2	3	3	5
10	0	1	3	3	5

Fig. 8.2.1. Chart of Sensitivity Test Results

Data from Table 8.2.1. was converted to percentages and plotted against observations time.

The number of fungal spores placed in each faecal mound is shown in the legend.



After six days there was a 60-80% chance of detecting trapping fungus in faecal mounds containing even a single spore, and this increased to 80-100% over the next four days.

8.2.2. Single Sheep Preliminary Trials

The penned sheep was drenched with various formulations of *Arthrobotrys oligospora* and its faeces collected for the next three days to be examined for the presence of trapping fungus.

8.2.2.1. Drenching with Spore Powder Suspension.

Forty grams of spore powder was suspended in 200ml water and administered to the sheep as a drench from a 350 ml glass bottle. Faeces were collected at 12 hour intervals for two days and examined for *A. oligospora* by the baited plate method. A portion of faeces was mixed with 0.1g spore powder and put onto baited plates as a control, to ensure that viable fungus would grow in the faeces.

Results

There was no trapping fungus found on any baited plates except for the controls, in which *A. oligospora* was detected after three days incubation. Viable spores were able to germinate and grow when placed in faeces, but no trapping fungus was detected on baited plates inoculated with any of the faecal samples

8.2.2.2. Drenching with Pelletised Conidia.

A suspension of fungal spores was mixed into 1% sodium alginate to make a thin paste which was formed into pellets by dropping from a pasteur pipette into 2% calcium chloride solution (see formulation tests). Approximately 65g of these pellets were administered to the sheep with 250ml water in a glass bottle. Faeces collected next day were used to set up seven baited plates.

No trapping fungus was found in any baited plates. Although the fungus remained viable after pelletising (see chapter 7), this treatment did not result in the passage of viable spores in faeces.

8.2.2.3. Drenching with Spores in Canola Oil Emulsion.

Conidia of *A. oligospora* were collected from mature plate cultures by washing with canola oil. Sixty ml of the oil (approximately 10,000 conidia/ml) was shaken with 250ml water to form an emulsion, and this was administered from a

glass bottle orally to the test sheep. Faeces were tested for viable fungus by the baited plate method.

No trapping fungus was found in any baited plates.

8.2.2.4. Passage of Fungus in Millet Grains.

Two tubs of whole millet grain fungus cultures were mixed with 400ml tap water and administered to the sheep from a 500ml glass bottle at 3:15pm. Faeces were collected from the calico bag at 9:30am and 3:30pm on the following day, and at 10:00am the next day. Seven baited plates were set up from each faecal sample. Plates were examined for trapping activity after three days, and again after three weeks.

Results

Millet grains were visible in faeces collected at 18, 24 and 42 hours after dosing. After four days' incubation, *A. oligospora* was present in three replicates of the seven from the 18 hr sample. No fungus appeared in any of the other baited plates in the following three days, and the plates were left on a laboratory benchtop. After three weeks the plates were re-examined and most contained *A. oligospora* . Scores for the 18 hr, 24 hr and 42 hr samples were 6/7, 4/7, and 6/7.

8.2.3. Effects of grain size and culture age on survival during passage through sheep

Gruner *et al.* (1985) reported survival of *Arthrobotrys oligospora* after whole grain millet cultures of the fungus were passed through the sheep. An attempt to repeat this in preliminary investigations gave a positive result, and it was thought that survival may have been due to rapid passage of the small whole grains through the rumen and survival of hyphae deep within the grain. If this were the case, the age of the culture could be important as older cultures may have no live hyphae left in the grain. The hypothesis was tested by feeding cultures of different ages, grown either on wheat or millet, to penned sheep.

Four 3-year-old Merino wethers were kept indoors in individual pens and fed once daily with a mixture of lucerne chaff and

sheep pellets. Sheep liveweights were 36.5, 37.5, 43.5 and 34.5 kg.

Arthrobotrys oligospora was grown on wheat or millet whole grain cultures in 250 ml polypropylene tubs. Grain cultures were incubated at 25°C for two weeks (young) or four weeks (old) before feeding to sheep.

Each sheep was fed one tub of grain cultured fungus mixed with its normal food on the first day of each experimental period. Faeces were collected in a calico bag attached to a harness on the animal, which was emptied each morning. Faeces collected during the five days following administration of the fungus were examined for the presence of viable propagules by the baited plate method. To ensure no carryover effects from previous treatments, experimental periods were started at 2 week intervals.

The experimental design was a Latin Square, each sheep receiving each of the four treatments during the four experimental periods. The treatments were:
1. YM (young millet) 2 weeks old millet culture.
2. OM (old millet) 4 weeks old millet culture.
3. YW (young wheat) 2 weeks old wheat culture.
4. OW (old wheat) 4 weeks old wheat culture.

The daily collection of faeces from each sheep was transferred to a plastic bag and mixed thoroughly. A sub-sample of about 60g was transferred to a smaller plastic bag and mixed and mashed by squeezing until it was reduced to a homogenous mass of crumbs. A spatula-tip (approximately 1 ml) of this material was placed in a star pattern on each of four water agar plates. This transfer was made in a laminar flow hood to prevent possible contamination by trapping fungi spores from other material in the laboratory. Bait nematodes were added and the plates incubated at 25°C. Plates were examined for trapping activity for two weeks.

Results

No trapping fungus was found in any faecal samples tested during this trial.

All 80 baited plates from the first trial period were examined frequently over a ten day period, and by the tenth day no fungus had emerged. By this time infective juvenile parasitic worms could be seen in the plastic bags containing the remainder of faeces collected during the trial period.

The sheep readily ate the grain cultures mixed with their normal diet during the first two trial periods, but one sheep was reluctant to eat the grain in the latter trials, carefully sorting it from the chaff and pellets in the feeding trough and leaving most of it uneaten until the day after dosing. However, all the grain was eaten before the next feeding time.

There were 320 baited plates examined regularly for signs of trapping fungus during this trial, and not one of them produced any trapping fungus. This was an unexpected result in view of the positive result obtained in the single sheep trials.

8.2.4. Survival of Fungus after Passage through Cattle

Three dairy cows, Lucy, an 8 year old in early lactation, Nancy, a 6 year old in late lactation, and Grace, a heifer, due to calve in five days, were each administered 40g spore powder containing 24 million conidia, mixed into their feed supplement at the morning milking. Faeces from each animal were collected in 250 ml polypropylene, lidded tubs both before the morning feed and again 8 hours later, at the evening milking. and at the next three milking times, which were the morning and evening of the following day, and the morning of the day after that.

Faecal samples were tested for the presence of nematode-trapping fungi by the baited plate technique. Three replicate plates and one control plate, using a portion of faeces to which spore powder had been added, were made from each faecal sample. Large quantities of the bait nematodes, infective juvenile

Heterorhabditis heliothidis, were added from a pasteur pipette, and the plates were incubated at 23°C. Plates were examined for signs of trapping activity two days later, and at intervals for a week afterwards.

Results

In the hours immediately following dosing, the cows appeared agitated and nervous. They were difficult to bring in for the evening milking and would not feed or allow the operator to milk them. Neither would they allow their calves to feed. The following day, calves were allowed to suckle and the cows were back to normal. After drinking from the cows, the calves also became agitated and nervous. Whether this was caused by the dose of fungus or the change of pasture which occurred on the same day is uncertain. There was no sign of this type of reaction to the fungus when it was fed to sheep.

No trapping fungus grew in any of the 27 baited plates prepared from faecal samples. The control plates (prepared from faeces to which spore powder had been added) all contained abundant *A. oligospora* , indicating that viable propagules could grow in the faecal material.

CHAPTER 9

GLASSHOUSE TRIALS

Introduction

A series of investigations were conducted with faeces placed on pasture swards grown in plastic tubs in a glasshouse. Faeces from a parasitised sheep were placed on the grass and the emergence of pre-parasitic nematodes monitored. There were three subjects of investigation;

1. The ability of *A. oligospora* to reduce pasture infectivity when inoculum is either added to the soil or sprayed onto faeces lying on the grass.
2. The concentration of spores needed, when applied as a spray, to get the maximum possible reduction of nematode numbers.
3. The effect of the time of spraying with fungus relative to the time of deposition of the faeces, on the ability of the fungus to reduce the numbers of surviving infective nematodes.

9.1. Materials and Methods

9.1.1. Production of Pasture Plots for use in Trials

A mixture of *Lolium perenne* and *Trifolium repens* (Victorian ryegrass and white clover) was sown in a standard potting mix made from sand, peat moss, dolomite, lime and Osmocote.
Grass was cut to two centimeters using a shearing handpiece as required during establishment of the pasture swards and before commencement of the trials (fig. 9.1.1.). Ten weeks were allowed for the establishment of a pasture mat in the tubs. Tubs were watered by hand-held spray as necessary. After the final harvest the tubs were emptied and cleaned before being re-filled with potting mix and sown in preparation for the next trial.

9.1.2. Sources of Contaminated Sheep Faeces

Contaminated sheep faeces for use in the first trial were supplied by DPIF Mt Pleasant Laboratories, courtesy Dr David Obendorf. These faeces contained 10,000 epg (eggs per gram) of *Trichostrongylus colubriformis* . During the collection period

faeces were kept at 4°C in a plastic bag. They were transported by bus in an insulated container with cooler bricks and refrigerated overnight before placement on pasture plots. Each tub received 40 g of faeces. Pellets were placed in contact with the soil and below the level of grass cutting by pressing them gently into the pasture mat (fig. 9.1.1.).

For the first spray concentration trial, four aged wethers were obtained from the Bridgewater saleyards and kept indoors in individual pens. One of these animals was passing 1,775 epg in its faeces, from a naturally acquired mixed infection, and faeces collected from this animal over a four day period were pooled and used for the second glasshouse trial at the rate of 35g per tub.

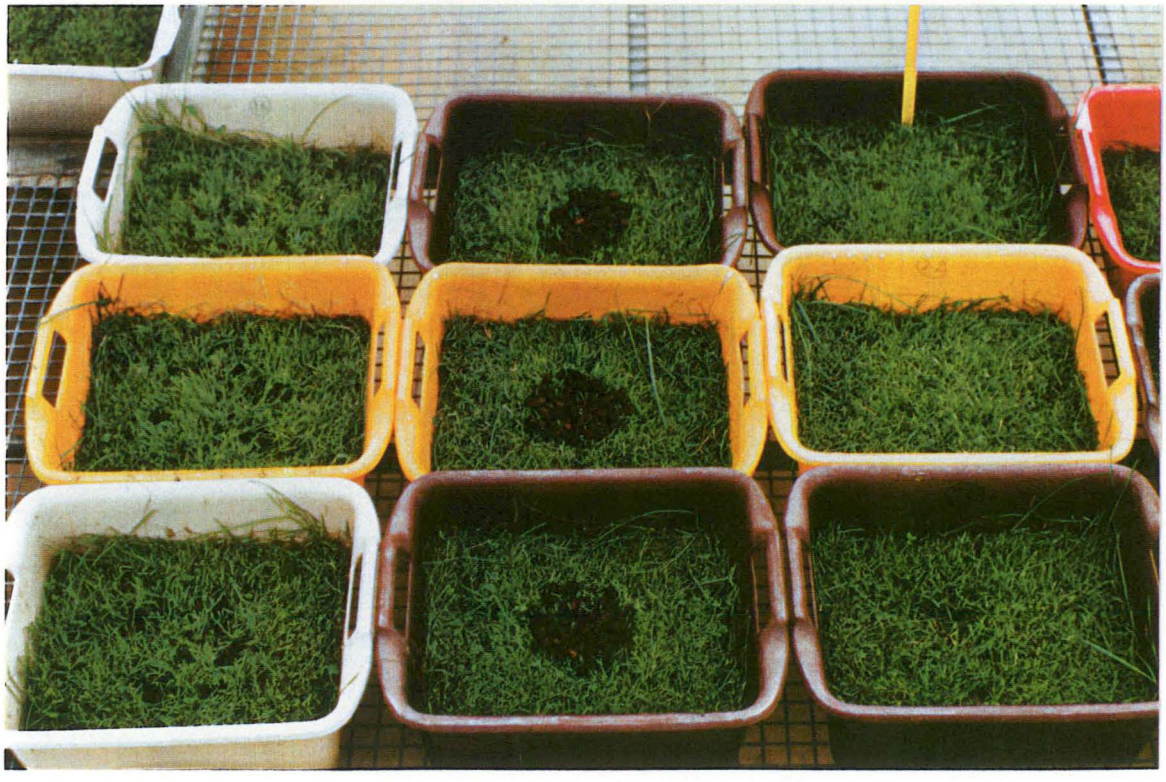
For the second concentration trial, faeces were collected from the same sheep which then had a faecal egg count of 1,100 epg. Each tub received 50g faeces from a pool collected over the previous four days. The third concentration trial used faeces from the same sheep, which then contained 1,000 epg, each tub receiving 60g of this material.

For the final concentration trial and the timing trial, faeces were collected from a 10 month old Suffolk wether with a naturally acquired mixed infection. Faeces from this animal contained about 8,000 epg but this could vary by 2,000 or more on a daily basis, depending on the volume of feed ingested by the donor. Since the timing trial required the production of uniform material over a three week period, this animal was hand fed and its faecal egg count monitored daily, prior to commencement.

9.1.3. Faecal egg counting method

The method described by Whitlock (1948) was modified as follows. Sub-samples of 1-2 g faecal pellets were weighed and placed in small plastic bags. An amount of water, ten times the weight of the pellets, was added and mixed with the faeces by squeezing with the fingers. When the faeces were dispersed in the water, a quantity of saturated salt solution equal to double the volume of water added, was mixed into the contents of the

Fig 9.1.1. Above: Tubs of pasture used in glasshouse trials
Below - Tubs with grass cut before faecal deposition showing placement of faeces and examples of tubs with faeces pressed into sward



bag. After leaving the bag aside for half an hour or more, to allow air bubbles to disperse, the mixture was stirred thoroughly by squeezing and fluid was transferred to a Whitlock slide for counting under a microscope.

Five replicate sub-samples were counted this way to estimate the egg counts in faeces used in the dose rate and timing trials.

9.1.4. Soil Inoculum

Arthrobotrys oligospora was cultured on sterile wheat grains in 250 ml polypropylene lidded containers. After four weeks incubation at 23°C, lids were removed and the sporulating fungus dried at 35°C. Dried cultures were passed through a hammer-mill, and the resulting powder used as soil inoculum. Potting mix was pasteurised and allowed to cool before adding fungal inoculum. A commercial fish and bark compost (OR90) was added to the potting mix before dividing it into two equal portions. Thirty tubs were filled from one portion and the other was returned to the mixer for the addition of fungal inoculum. About 500 g powdered fungus cultures was added to the potting mix, which was then placed into another 30 plastic tubs.

The presence of the fungus was confirmed one week prior to the trial by placing soil samples on baited agar plates. Ten soil-inoculated tubs and ten without soil inoculum were selected at random and samples of potting soil removed for testing on baited plates. All ten samples from inoculated tubs produced trapping hyphae after three days. None of the samples from the non-inoculated tubs grew any trapping fungus on baited plates.

9.1.5. Spray Inoculum

Conidia were removed from dried grain cultures by mashing the grain with a stiff bentonite slurry and then washing the suspension through a sieve with tap water. This separated the spores from the spent grain and formed a dense fungal suspension of about 300 million spores per ml. Spores harvested in bentonite slurry were used to prepare sprays for the inoculum placement trial and the first three spray concentration trials. For

the fourth spray concentration trial and the timing trial, spores were collected from 20 mature petri dish cultures by mixing with tap water and mashing by hand in a plastic bag. The resulting suspension was passed through a sieve (22 mesh) and spores were counted in diluted samples in a Whitlock slide. Bentonite was omitted from the sprays used in these trials because at the high spore concentrations used, it may have acted as a thickener or interfered with the moisture characteristics of the faecal pellets.

The density of the spray used in the trial comparing soil and spray inoculum effects was 34,000 conidia/ml. In the concentration trials, spray densities were as reported. In the timing trial, a single concentration of 10^6 conidia/ml was used.

9.1.6. Recovery of infective juvenile nematodes from pasture plots.

Grass was cut at weekly intervals from all tubs in the first three trials, and only once in the final trials. From the results of the earlier trials it was clear that most of the infective larvae had migrated to the grass after six weeks and a single harvest at this time would provide sufficient data for analysis. It was considered that the death rate of infective larvae on the grass during this time would have a negligible effect on the outcome (Leathwick *et al.* 1992)

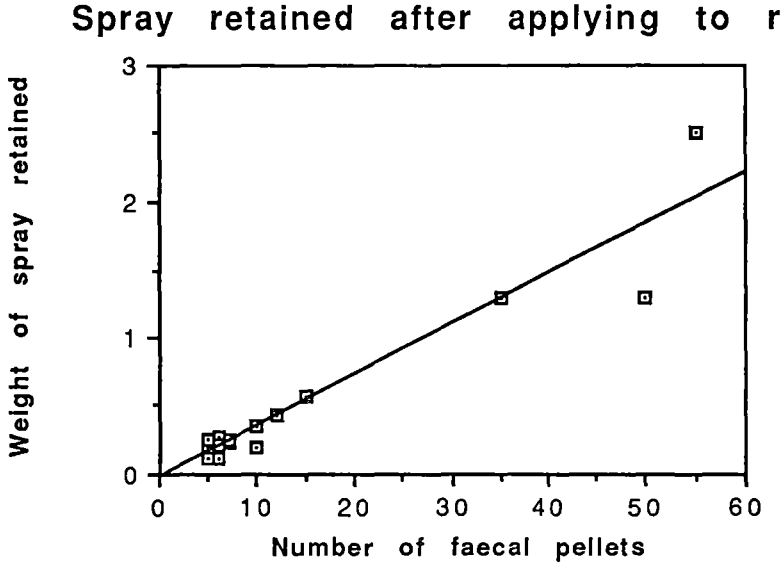
A portable shearing machine was used for cutting grass from the pasture tubs. Clippings were collected on a sheet of plastic and transferred carefully to a labelled paper bag for transport to the laboratory. In the laboratory, the grass clippings from each tub were weighed and transferred to a 2 l enamel container. Five hundred ml tap water and two drops of Tween 80 were added to each container, and the clippings allowed to soak overnight. The next day the contents were vigorously stirred before decanting the liquid into a 600 ml beaker through a kitchen strainer. Two 5 ml aliquots were withdrawn while stirring the contents of the beaker, and placed on a marked petri dish for examination under a dissecting microscope.

Nematodes were counted using a hand-held counter to record worms sighted while scanning the water sample systematically with the aid of a grid scratched onto the bottom of the petri dish. Nematodes which were greatly different in size, swimming action or morphology (eg those with an obvious mouth) were not counted. There were very few such worms encountered; the majority were vigorously wriggling, ensheathed, infective juvenile parasitic nematodes.

9.1.7. Spray Retention on Faeces

Before embarking on the spray trials, small quantities of faecal pellets were sprayed with water to determine the volume held on the average pellet when sprayed to runoff with water. Faeces were placed on a flywire mesh and weighed. They were then sprayed with water, the mesh base blotted dry, and reweighed. Results are summarised in figure 9.1.1.

Fig. 9.1.1. Spray Retention on Faeces.
Faeces came from a bagged sheep maintained on a diet of chaff and sheep pellets.
Water was applied by hand-operated spray until excess ran off faeces.



Obviously, the volume of water held by a pellet will vary with the state of dryness of the pellet as well as its size and the duration of spraying. These results were obtained using fresh

faeces from the donor sheep so that an approximate estimate could be made, of the number of conidia per gram of faeces delivered by spraying.

The total number of faecal pellets used was 245, weighing a total 75.5 g dry, and 85.9 g wet, with the average amount of water retained per pellet being 0.0424 ml. Average pellet weight was 0.308 g. The amount of water retained per pellet was approximately the same regardless of the number of pellets in the group sprayed.

Assuming suspended conidia remain in the retained water, the required inoculum per gram of faeces can be applied by adjusting the concentration of conidia in the spray. Grønvold (1985) reported 99% reduction in third stage larvae emerging from faecal cultures when conidia of *A. oligospora* were added at the rate of 2,500/g, and a 70% reduction from an inoculum rate of 250/g. Assuming the average weight of a faecal pellet is 0.3 g, the spray concentrations needed to duplicate those inoculum rates are 18,000 conidia/ml and 1,800 conidia/ml. Even a very low rate of fungal inoculum may reduce the survival rate of worm larvae (Waller and Faedo, 1993), so conservative concentrations were applied in the first spray trials. Results from these trials indicated higher concentrations were needed, and a wide range of concentrations was tested over the four trials.

9.2. Effects of Soil and Spray Inoculum on Pasture Infectivity

Faeces from an infected sheep were placed on small pasture plots grown in a glasshouse. Some plots were inoculated with *Arthrobotrys oligospora* either in the soil, or by spraying after faecal deposition, or both, while others remained untreated as controls. Grass cut from the plots was examined for infective larvae over the following six weeks.

9.2.1. Experimental Design

There were four treatments:

1. Fungus applied as a spray to the faeces.
2. Fungus applied as soil inoculum.

3. Fungus applied as both soil inoculum and a spray to faeces.
4. No fungus application.

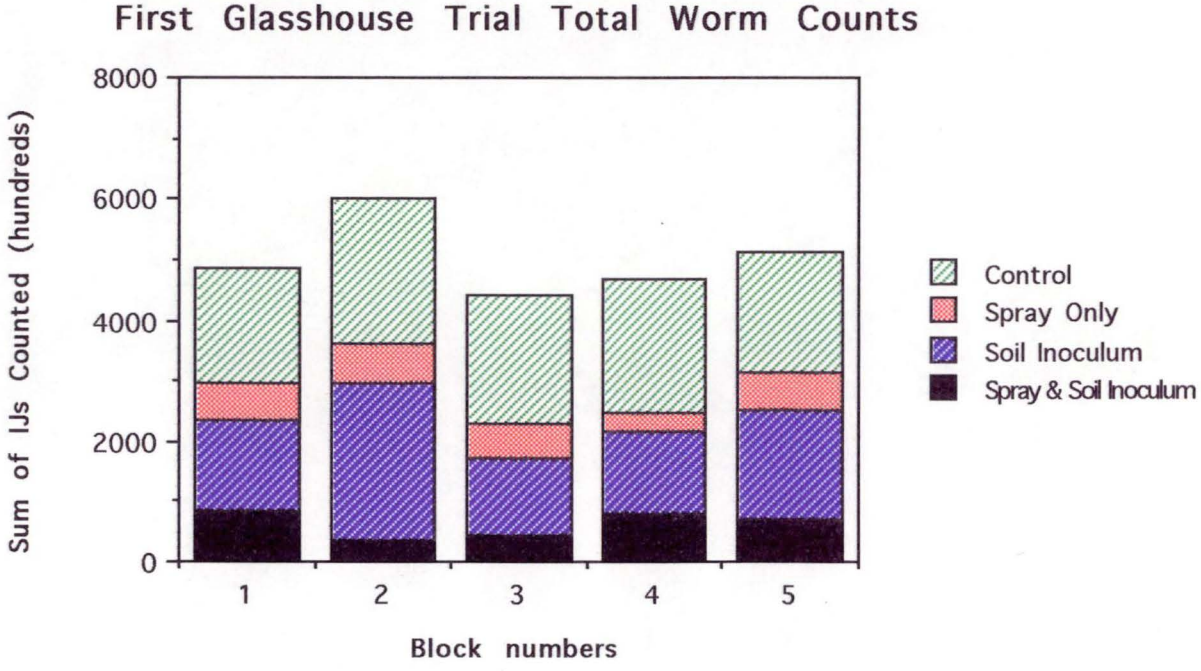
Treatments were allocated randomly to 60 tubs divided into five blocks containing three replications of each treatment. Each block occupied a separate location in the glasshouse, and the tubs were re-randomised within each block at 2 weekly intervals. Blocks were harvested at different times of the day, and some were watered before harvesting to determine the effect of these factors on the numbers of nematodes recovered.

9.2.2. Results

Tables of data from the glasshouse trials appear in the appendix. Analysis of variance showed that differences between treatments could be detected within blocks with only three replications of each treatment, but variance within treatments was large. There was no significant difference between blocks in the final outcome although data from weekly harvests occasionally ranked treatments differently within different blocks. For example, in week 1 plots with spray and soil inoculum had higher counts than those with soil inoculum only in block 4, while in all other blocks spraying reduced counts more than soil inoculum did. These anomalies disappeared when all weekly data was pooled. However, for future trials, a single block containing five replications of each treatment was used.

The total numbers of nematodes recovered over the full period of the trial are shown in Fig. 9.2.1. Spraying reduced nematode numbers to 24.8% of the controls on average, though this varied from 15% in block 4 to 33% in block 5. Soil inoculum reduced counts to 75% of the controls on average, but this varied from 46% in block 3 to 92% in block 5 and 110% in block 2, and was not statistically significant.

Fig. 9.2.1. Effects of Fungus as Soil or Spray Inoculum.
Columns show the total numbers of infective juveniles (IJs) recovered in each block, with the contribution from each fungus treatment appearing in a different colour.
Blocks contained three replicates of each treatment.
Inoculum was applied in a spray or mixed with the soil as shown



by the legend.

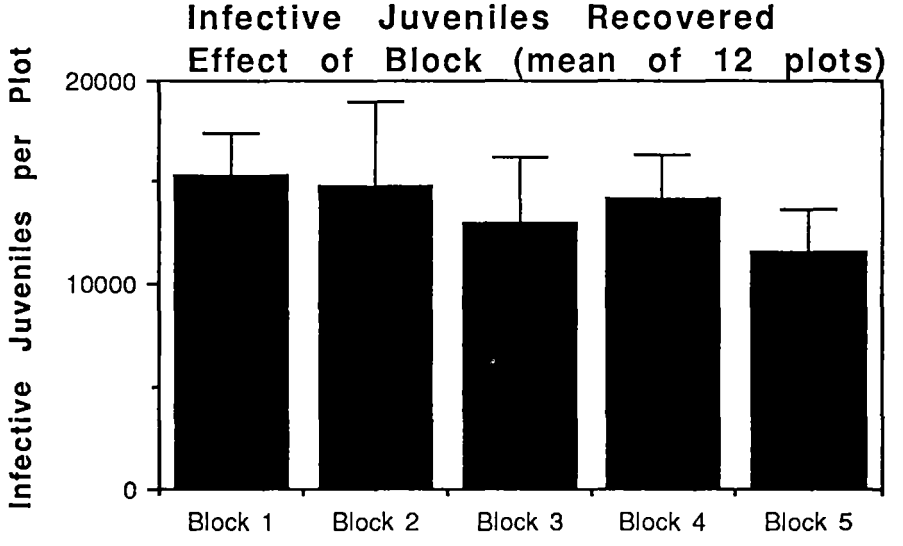
The recovery of nematode larvae from grass cut from plots was similar among the blocks, and the effects of treatments were similar within each block. The data above is the sum of all nematodes counted over the experimental period. Differences between blocks were insignificant, allowing the block effect to be removed from the analysis.

At the first harvest, there was concern that diurnal migration may affect the numbers of nematodes recovered, so the grass was cut at different times for each block to determine whether this affected nematode recovery.The harvest time of each block was as follows:
Block 1. 9:00 - 11:00am.
Block 2. 2:15 - 3:15pm.

Block 3. 4:00 - 5:00pm.
Block 4. 7:00 - 8:15pm.
Block 5. 8:15 - 9:15pm.
Blocks 4 and 5 were watered at 5:00pm.

Although there was a small decline in the numbers of infective juvenile nematodes harvested later in the day, differences between blocks were not statistically significant, as can be seen in Fig. 9.2.2. If watering had any effect on nematode numbers, this, also, was insignificant.

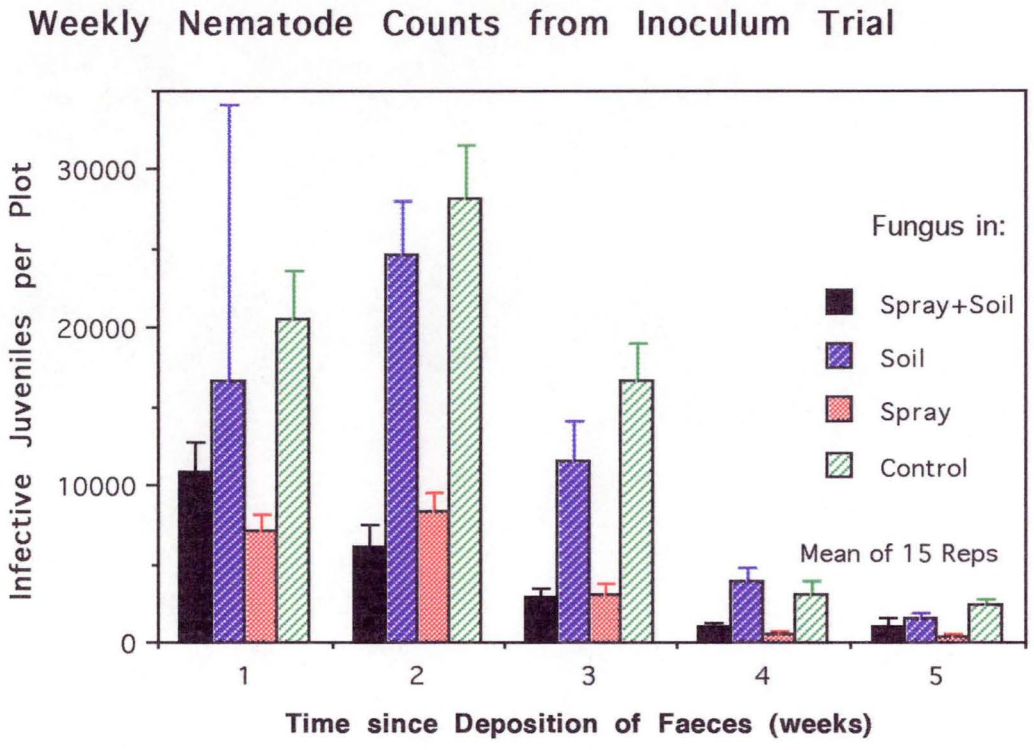
Fig. 9.2.2. Nematode Yields after 1 Week
Grass was cut from blocks at different times of the day



Two blocks were watered before harvesting
Grass was soaked overnight and infective nematodes counted
The mean and standard deviation of the 12 plots within each block are plotted below

A summary of weekly results can be seen in Fig.9.2.3, where the effect of spraying is most obvious in weeks two and three.
Sprayed plots had significantly reduced nematode counts at each weekly sampling time throughout the experiment.

Fig. 9.2.3. Effects of Fungus Placement on Survival of Infective Juveniles
Counts are the number of IJs washed from cut grass.
Weekly counts are means and std. deviations of 15 reps.
Fungal inoculum placement shown in legend.
Controls received no fungal inoculum.



Most of the worms had migrated to pasture herbage after three weeks, with the majority moving in the first two weeks. The faeces used in the trial were collected over several days and spent a day in transit before being placed on the pasture plots, so it is possible that some development had taken place before the trial began. This could explain why the fungus had less effect during the first week than it did later.

The presence of fungus in the soil had little effect on nematode survival. One week before commencement, samples were taken from ten of the inoculated tubs and the presence of *Arthrobotrys oligospora* was confirmed using baited petri plates. There may have been some effect on migrating larvae that entered the soil, but if so they were too few to affect the numbers arriving on the

grass. The results from block 3, where plots with soil inoculum averaged 46% of the numbers of nematodes recovered from the controls, suggest that soil inoculum can reduce nematode survival somewhat, but over all blocks, soil inoculum numbers averaged 75% of the control nematode numbers, and the reduction was not statistically significant.

9.3. Concentration Trials

Contaminated faeces were placed on pasture plots and subjected to sprays containing various concentrations of conidia of *Arthrobotrys oligospora*. The numbers of infective larvae migrating to the grass were monitored over six weeks

9.3.1. Experimental Design

These trials contained five replications of each treatment in a single randomised block. There were four concentration trials, using different ranges of spore densities.

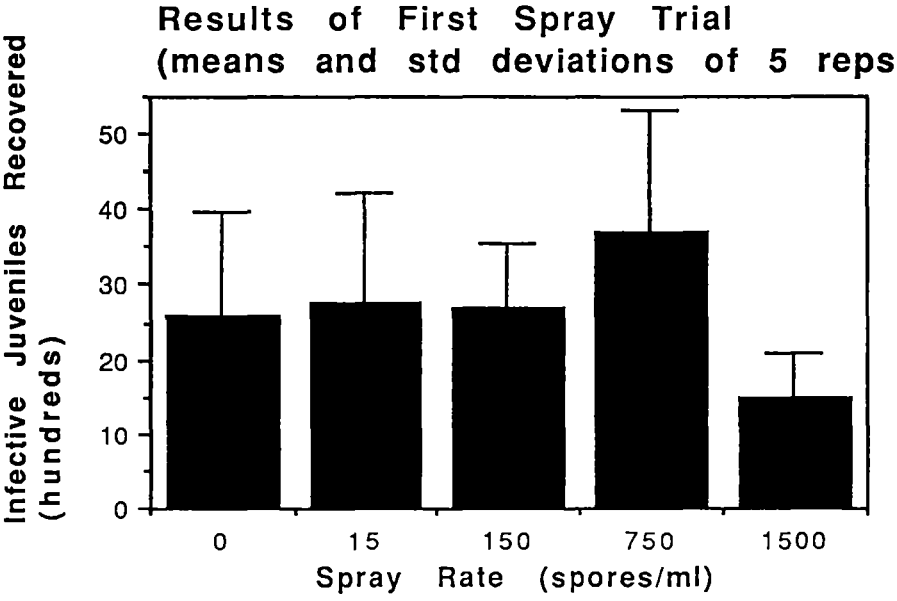
Fungus was applied at a different range of concentrations in each of these trials, as follows:

1. 0, 15, 150, 750, 1500 spores/ml.
2. 0, 100, 500, 1,000, 10,000 spores/ml.
3. 0, 100, 500, 1,000, 10,000 spores/ml.
4. 0, 10^2, 10^3, 10^4, 10^5, 10^6 spores/ml.

9.3.2. Results

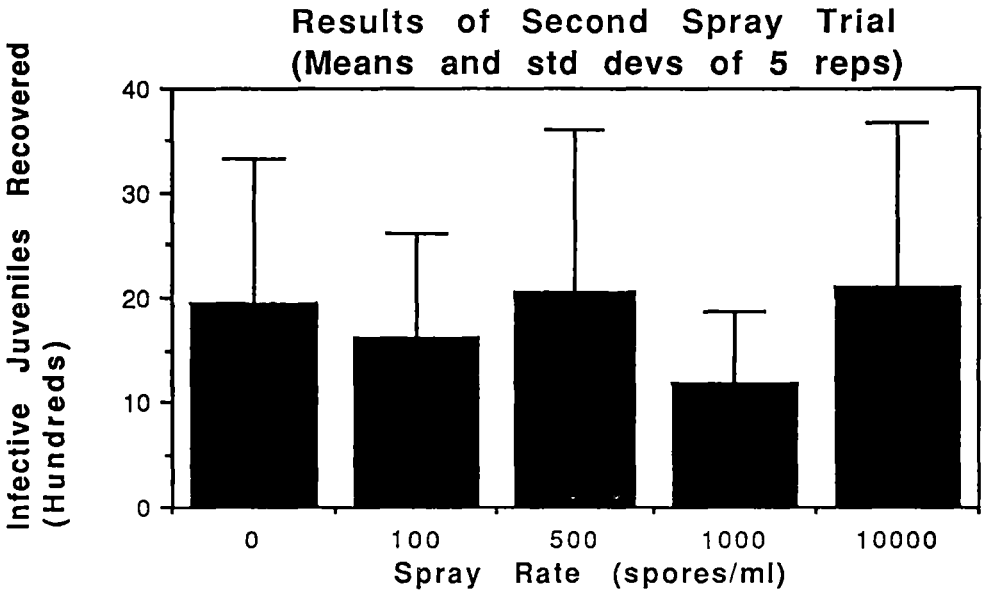
The first trial failed to reproduce the reductions in infective juvenile (IJ) nematode numbers obtained in the inoculum trial. Spray rates were much lower than the 34,000 conidia/ml used in the inoculum trial spray. At the highest rate (1,500 conidia/ml) a small reduction in nematode count occurred, but this was not statistically significant and was overshadowed by an increase in numbers from the plots sprayed with 750 conidia/ml. The results are shown in the chart in fig.9.3.1. on the next page.

Fig. 9.3.1. Infective Nematodes Recovered During First Spray Trial.
All plots were sprayed once on the day of faecal deposition. Grass was cut and worms counted after 1, 2, 3 and 5 weeks. Results shown are total of worms recovered during trial period. There were 5 plots sprayed at each of the different rates



Generally, survival of the nematodes was very low, with fewer than 3,000 worms recovered from control plots. Plots received 35g faeces containing 1,800 epg, a potential of 63,000 infective larvae per plot. Mortality was greater than 95% without predation by fungus. This may have been caused by high temperatures in the glasshouse and possibly desiccation during the first weeks of the trial period. These conditions may also have affected survival of the fungus. The apparent reduction at the highest concentration prompted the use of even higher concentrations in the following trial.

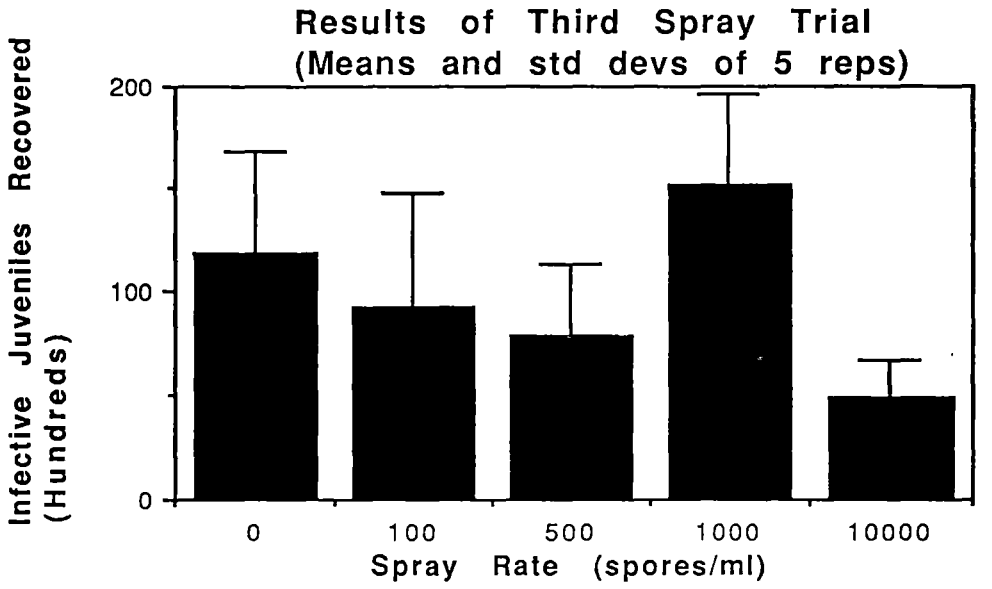
Fig. 9.3.2. Infective Nematodes Recovered During Second Spray Trial
All plots sprayed on the day of faecal deposition.
Five plots were sprayed at each concentration.
Grass was cut and worms counted after 2 and 4 weeks.
Mean totals of IJs per plot at each concentration are plotted



below

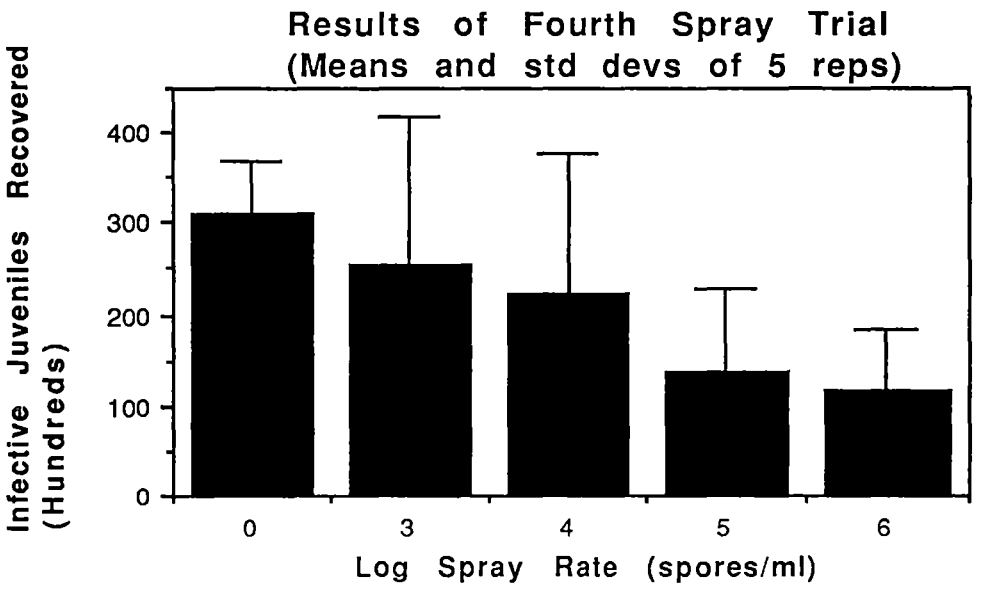
In the second trial, plots received 50g faeces with 1,100 epg, a potential 55,000 infective juveniles (IJs) per plot. Numbers of IJs recovered ranged from 400 to 4,300 with an average over all plots of 1,750, a mortality rate of 97%. At the highest concentration, the average was 2,070 IJs per plot, just slightly higher than that of the controls (1,920 IJs/plot). Once again, abiotic factors had more influence on nematode survival than predation by fungus.

Fig. 9.3.3. Infective Nematodes Recovered During Third Spray Trial.
All plots sprayed on day of faecal deposition.
Five plots were sprayed at each concentration.
Grass was cut and worms counted after 3 and 6 weeks.



In the third trial, survival of IJs in the control plots averaged 18% at 11,800 per plot. These plots received 60g faeces with approximately 1.000 epg, giving a potential of 60,000 IJs per plot. Significant reductions were obtained, with the highest spray concentration reducing numbers to an average of 4,800 per plot, a survival rate of 8%. Paradoxically, plots sprayed at the second highest concentration averaged 15,100 IJs, a survival rate of 25%. Without this odd result, there appears to have been a dose-related response to the fungus (see fig. 9.3.3.). It is unlikely that the presence of the fungus would have resulted in the increase in worm numbers seen in those plots. Perhaps a mistake during the preparation of spray dilutions occurred, and no fungus was sprayed onto these plots. This, however, is conjecture. The results obtained throw doubt on the significance of the reductions due to the fungus.

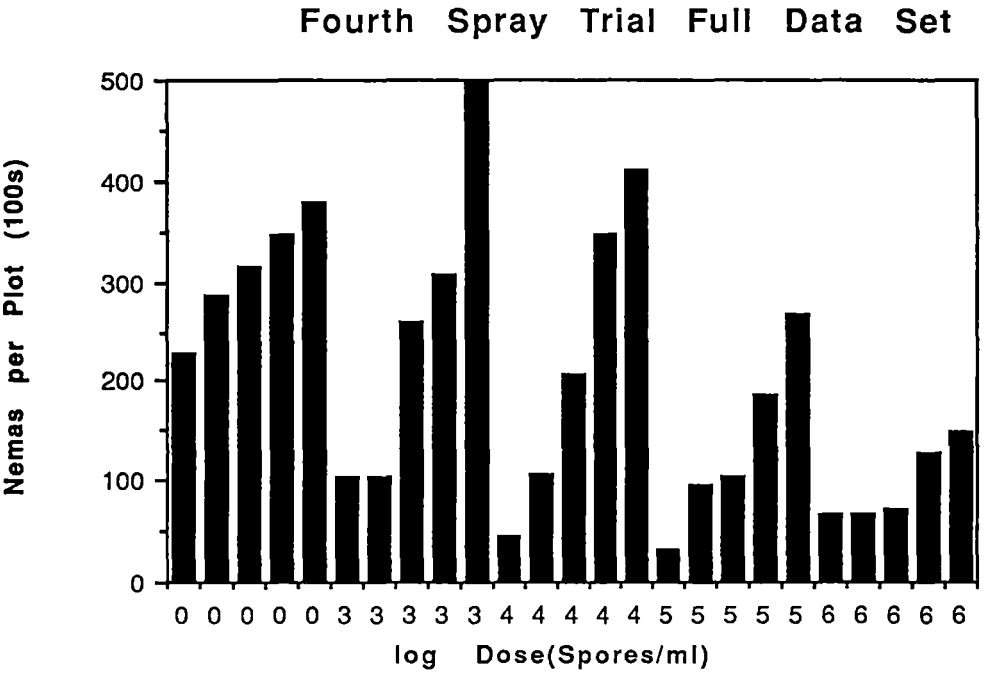
Fig. 9.3.4. Nematodes Recovered During Fourth Spray Trial
All plots were sprayed once on the day of faecal deposition
Five plots were sprayed at each concentration.
Grass was cut and worms were counted once after six weeks.



In the fourth trial, both the concentrations and the faecal egg count were increased. Plots received 60g faeces with 8,000 epg, giving a potential of 480,000 IJs per plot. The average number of IJs recovered from control plots was 31,060, a survival rate of 6.5%. Fungus at the rate of 10^6 spores/ml reduced the average count to 9,500 per plot, a survival rate of slightly less than 2%. A dose-related response to the fungus is evident in figure 9.3.4. This was statistically significant at the $P<0.05$ level.

Clearly, the concentrations used in the first two trials were too small. The spray density of a million conidia per ml, used in the fourth trial, reduced average nematode counts more than lower concentrations, but an examination of the full data set, shown in fig. 9.3.5. shows that equally low worm counts appeared at all concentrations, but occurred in fewer replicates at lower rates. In other words, where the fungus failed to reduce nematode numbers it may have failed to establish, or had colonised only a small portion of the faeces.

Fig. 9.3.5. Full Data Set from Fourth Spray Trial
All plots were sprayed once on the day of faecal deposition.
Five plots were sprayed at each concentration.
Grass was cut and worms were counted once after six weeks.



Two of the replicates receiving 1,000 and 10,000 spores per ml showed similar nematode counts to those sprayed at the highest concentration. The response to fungus could be related to the volume of faeces colonised by hyphae, and the results shown above may reflect this. Perhaps, at higher spray concentrations, the probability of colonisation is increased, but the results of successful colonisation are similar, no matter what the inoculating dose.

Some of the variability may have been due to differences in the age of faeces used in the trial. Faeces were collected over several days and stored in a refrigerator before use. Pooled faeces were mixed before deposition, but it is still possible some plots received a preponderance of faeces which were older than most. The results of the timing trial show that faecal age at the time of spraying has a significant effect on the ability of the fungus to reduce worm survival.

9.4. Timing Trial

To determine the effect of timing, faeces were placed on the pasture on the day of collection, this varying from up to 14 days before, to 7 days after the time of spraying the pasture. Grass was cut from all plots six weeks after spraying, and the numbers of infective larvae recovered from the clippings determined as previously described.

9.4.1. Experimental Design

There were 5 different times tested for deposition of infected faeces relative to the time of spraying. These were:

- 14 days before spraying.
- 7 days before spraying.
- 3 days before spraying.
- on the same day as spraying.
- 3 days after spraying.
- 7 days after spraying.

There were five replications of each treatment in a single randomised block.

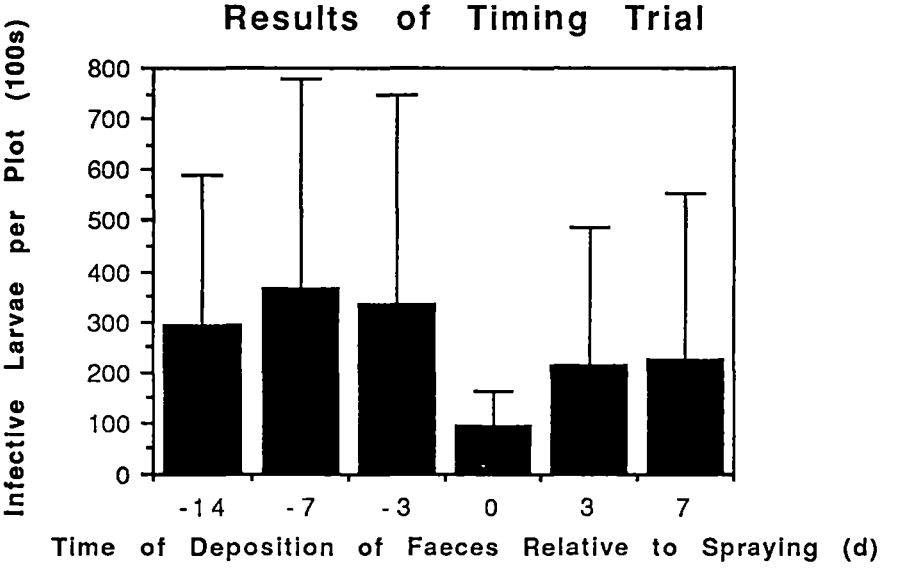
Each of five pasture tubs received 60g of fresh faeces from the donor sheep on each deposition date. All tubs were sprayed once with a suspension containing 1,000,000 conidia per ml of the fungus, *Arthrobotrys oligospora*.

9.4.2. Results

The time of deposition of faeces relative to spraying had a significant effect on nematode survival. There was no reduction in the number of infective larvae recovered from plots sprayed only three days after deposition of faeces, and none in plots sprayed later than this. Faeces used in this trial had similar faecal egg counts to those used in the fourth spray trial, so the potential number of IJs per plot was about 480,000. Reductions in the plots sprayed the same day as deposition were similar to those in the fourth concentration trial, with survival reduced to 2%. Otherwise, survival was similar to the control group in the fourth concentration trial, at about 6.5%.

The averaged results of this trial show the effect of timing dramatically. These results are shown in fig. 9.4.1.

Fig. 9.4.1. Infective Nematode Counts in Timing Trial
Faeces were collected from donor sheep on the day of deposition.
All plots were sprayed on day 0.
Grass was cut and worms were counted after six weeks.
Results shown are the means and standard deviations of 5 reps.

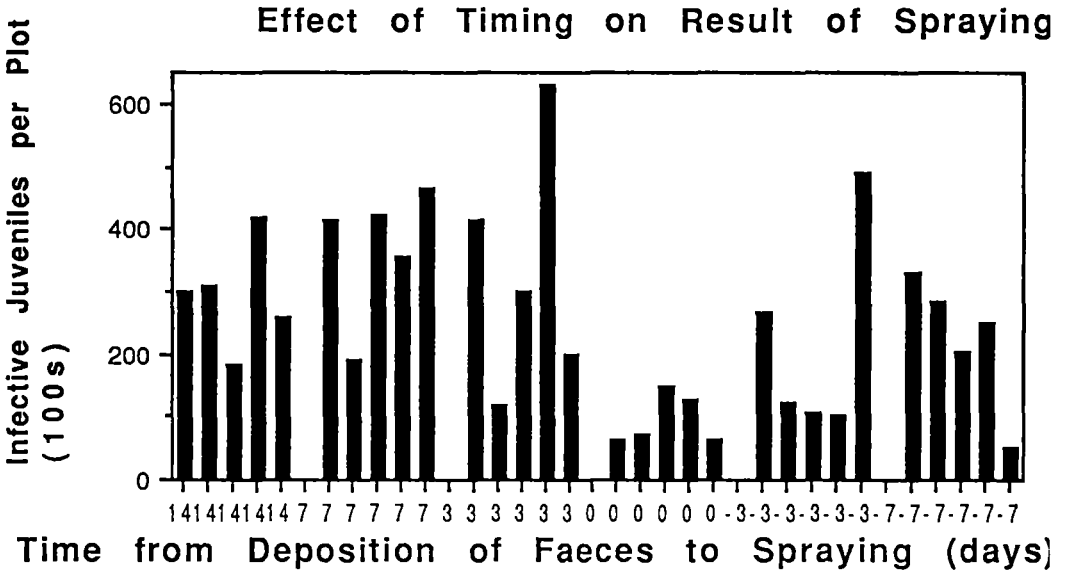


An interesting feature of these results is the large variation within treatments. This was probably the result of differences in the initial establishment of fungal colonies in different pasture plots. In these treatments the fungus seems to have increased the variability of survival rates between plots rather than reduced the average numbers of infective larvae surviving. This can be seen in figure 9.4.2, in which the results from all plots are shown.

There was a reduction in the number of nematodes arising from three of the plots in which faeces were placed three days after spraying but there was great variation between replicates of this treatment. No reduction can be claimed in faeces deposited seven days after spraying, yet one replicate of this treatment group had the lowest number of infectives of all.

Fig. 9.4.2. Full Data Set from Timing Trial

All plots were sprayed with 10^6 spores/ml on day 0.
Fresh faeces were placed on 5 plots on days 14, 7 and 3 before spraying and on days 3 and 7 after spraying, as well as on day 0. Grass was cut and worms were counted six weeks after spraying.



The results show that for spraying to be effective, it must be applied soon after the deposition of faeces, certainly within three days. There may have been some residual effect of spraying, but it was inconsistent after three days and negligible after seven days.

CHAPTER 10

DISCUSSION

10.1. Farm Soil Survey

Reports on the distribution of trapping fungi in Australian farm soils are few (Larsen *et al.* 1994), and the role that these predators might play in the ecology of the free-living stages of animal-parasitic nematodes is unknown. Soprunov (1958) considered their disribution to be global and was able to isolate a number of different species from many of his soil samples. However, he shared Duddington's (1957) observation that our ecological knowledge of these predators was limited to reports on their frequency of isolation within particular environments. For example, Dreschler (cited by Soprunov, 1958) found many of his isolates in samples of forest soil covered with a layer of decaying foliage. Soprunov (*ibid.*) suggested that "the habitat of predaceous hyphomycetes is characterised by the following ecological conditions:

a) high moisture (the substrate should not dry up);
b) presence of organic compounds (products of cellulose decomposition);
c) temperature range from 15 to 30°C, without extreme fluctuations;
d) pH of the medium close to neutral;
e) access of oxygen, i.e., a sufficiently high redox potential of the medium;
f) presence of nematodes or other small creatures captured by fungi."

Soprunov (*ibid.*) noted that predaceous fungi were often found in manure and fresh dung of horses and other farm animals and mentioned Duddingtons observation (cited *ibid.*) that the species isolated from manure varied with the course of time. However, he found there was insufficient evidence to establish the bioceonotic relationships of these fungi.

Gray (1984) found that a predator-prey relationship existed between the nematode population in activated sludge at sewage works, and the endoparasitic fungi *Meria coniospora* and *Catenaria anguillulae* . However, net-forming predators found in the same system did not appear to affect nematode populations. Other investigators such as Pandey (1973) and Grønvold *et al.* (1985) have demonstrated the susceptibility of animal-parasitic nematode larvae to fungal traps and shown that these nematodes stimulate trap formation, but no evidence of a relationship exists, other than reports of the isolation of predaceous fungi from animal manure.

The results presented here suggest that trapping fungi have not become associated with sheep grazing in Tasmania. Of approximately fifty paddocks sampled, there were fewer than twenty in which positive samples were obtained. No predatory fungi were found even in soil samples taken from paddocks in which sheep suffered chronic parasitism.

If a particular nematophagous fungus had become associated with sheep parasites in Tasmania, it would have been expected to have been isolated repeatedly from soils from a number of farms. However, such was not the case. Where they were found, predatory fungi seemed to be localised strains with no indication that any predatory fungus has become adapted to sheep farms or that there had been a spread of predaceous fungi between farms associated with movements of sheep. It is possible some fungi were missed if they were located below the sampling depth; from such a depth however, it is unlikely that the fungi would colonise faeces, and thus they would not play a role in the ecology of nematode parasites.

This survey was limited in the range of climates pertaining to the properties sampled. The districts covered were all relatively low rainfall areas (from 100 to below 50 cm annually) and represent the major wool growing areas of Tasmania. Parasitic nematodes are a serious problem for graziers in these areas, and the fact that fungi were present at some locations would indicate that climate was not a limiting factor for their survival.

The occurrence of trapping fungi in recently cultivated soils at Cullenswood, Rostrevor, Malahide and Springvale, may have been influenced by the aeration and possibly increased nematode activity following soil disturbance. Decaying vegetable matter would provide a substrate for free-living microbivorous nematodes, and a surge in their population could have stimulated the nematophagous fungi. It is also possible that cultivation had brought them to the surface. Bailey and Grey (1985) found that net-formers were the only nematophagous fungi isolated from below the top 10 cm of soil. Predatory fungi were relatively infrequent in soil samples taken from the top few centimetres in this survey. It is interesting that most of the soils in which *Arthrobotrys* was found had been recently cultivated.

It was interesting to find that predatory fungi were present in the black soil side of the "Strawberry" paddock at Leverington, but not in the sandy soil on the other side. This paddock was remarkable in that the pasture on the black soil side was mostly eaten out by larvae of the scarabaeid beetle, *Adoryphorus couloni*, while pasture on the sandy side remained intact. It is tempting to speculate that insect-pathogenic nematodes, such as *Heterorhabditis bacteriophora*, were controlling pasture grubs in the sandy soil, but the presence of *Dactylella* in the black soil may have protected the beetle larvae from parasitism. Why the fungus was not found on both sides of the paddock remains a mystery. Although predatory fungus was found in heavy black soils at some locations, it was also found in coarse sandy soils and light silty loams, and was not associated with any particular soil type. Possibly the sandy soil was a better environment for nematodes; for example, Boag and Lopez-Llorca (1989) found that cereal fields infected with *Heterodera avenae* tended to be those with a higher sand fraction. In a survey of nematophagous fungi and nematodes in cereal fields and permanent pastures in Eastern Scotland, these authors found *A. oligospora* to be more prevalent in cereal fields than in permanent pasture, while larger populations of plant-parasitic nematodes were found in permanent pastures than in cereal field soils.

The species most frequently found in pasture soils belonged to the genus, *Dactylella*, with *Arthrobotrys* spp. showing up in recently cultivated soils under crops. When isolates were compared for their growth rates on artificial media, there were differences between isolates of similar species from different locations. Isolates varied in their trapping behaviour, both in the distribution of traps on hyphae and the intensity of trapping. Zymograms prepared from isolates (results not reported) confirmed that they were different. Unfortunately, some of the isolates were lost before further zymograms were prepared and the original ones were not considered clear enough to be used in this report. The evidence was sufficient to suggest that isolates originating on different farms were different enzymatically as well as in growth rate and trapping behaviour. This observation may well be worth following up as a subject of further investigations.

Comparison of all isolates showed that the strain of *Arthrobotrys oligospora* (A1) used throughout this project was superior in trapping activity on agar, to any other fungus tested. Even though two strains of *Arthrobotrys* grew more rapidly on laboratory media, they were far less predaceous. One of the *Dactylella* spp. was predaceously active but grew slowly and sporulated poorly on laboratory media. There was a possibility that the ecology of pre-parasitic larvae on Tasmanian sheep farms might be altered by introducing a predator with a degree of vigour to which larvae had not previously been exposed. Questions such as "How could it be introduced?", "What reduction in nematode survival could be expected?" and "How often would it be need to be applied?" were the subject of the remainder of the investigations.

10.2. Nematode/Fungus Interactions

10.2.1. Density Experiment

Results of this experiment fall into two categories: first, the initial response, when the fungus commenced predaceous activity, provided there were sufficient nematodes to initiate trapping; and second, the longer term response, when trapping activity was influenced more by the size of the prey population than the initial fungal inoculum density.

During the first week fungal inoculum size was important, with complete capture of nematodes occurring only at the highest inoculum rate. The quantity of 50 bait nematodes did not stimulate trapping at any fungal inoculum size, suggesting that the threshold for stimulation of trap induction was not reached. This should not be surprising considering the results of Wyborn *et al.* (1969), who found that *Arthrobotrys oligospora* required the addition of 1,000 bait nematodes to plate cultures for trap induction. The implications of this for the control of pre-parasitic larvae in animal faeces are that low nematode populations may not be subject to predation. Unless trapping is induced by parasite densities below the threshold for economic damage to livestock production, predatory fungi will be incapable of preventing losses due to parasitism, although they may prevent severe epidemics. Just what density of pre-parasitic larvae in faeces represents a threat to livestock production will vary between nematode species and livestock enterprise, being affected by many factors such as stocking rate, seasonal grass growth, abiotic mortality factors and host susceptibility. However, if a faecal egg count of 500 eggs per gram is considered to be a threshold for the decision to use anthelmintics (as it was in the "Weaner Watch" program, cited in chapter 5), then one would hope that a fungal predator would begin to operate before this level is reached.

The density of prey required for trap induction within faeces may be different to that required on agar plates. Nordbring-Hertz and Brinck (1974) found mineral salts altered the response to inductive peptides, and Dackman and Nordbring-Hertz (1992)

suggested animal manure stimulated spontaneous trap formation in the germinating conidia of *A. oligospora.* Jaffee *et al.* (1992) found the number of traps formed on hyphae growing in soil extracts varied between extracts from different soils, and this variability should be taken into consideration when interpreting the results of this experiment. Wyborn (1969) found a threshold density of 5,000 bait nematodes per petri dish was needed to induce trap formation in some fungi, but 500 was enough to stimulate trapping in the strain of *A. oligospora.* used in this project. Given that trapping behaviour in soil or faeces may vary considerably from that observed in a petri dish, the nematode density required to elicit a response on agar may not be necessary to stimulate a similar response within soil or faeces. These experimental results may indicate the way this fungus responds to fluctuating nematode densities, but inferences drawn from attempts to compare experimental prey densities to those found in the pasture environment should be treated with caution.

By the end of the observation period, nematode population size was by far the most important factor affecting the efficiency of capture, with large populations being almost completely destroyed, while smaller populations were less severely predated, allowing greater numbers of nematodes to survive.

Although 5,000 larvae were enough to stimulate a flurry of trapping in the first week, subsequent additions of this amount did not sustain the intensity of trapping. Even when initial nematode numbers were 500, the increase to 5,000 did not have the effect of an increase to 50,000. This suggests that the threshold for trap induction may increase with colony age, as suggested by Barron (1977).

The implications for fungal activity in relation to biocontrol are several. Colonies growing in the presence of low numbers of nematodes may not become predatory unless numbers increase dramatically. When greater numbers of prey are present, colonies will trap them actively for a short time, but unless nematode numbers continue to rise, trapping activity will decline. Thus, the proportion of the nematode population

removed by predation will vary, depending on nematode numbers, fungal inoculum size and the timing of the arrival of the prey population, relative to the germination of fungal spores.

10.2.2. Trapping vs Hyphal Growth

The efficiency of nematode predation by fungi growing in faeces may be affected by the extent to which hyphae colonise the substrate. Conidia germinating in faeces may form traps spontaneously (Dackman & Nordbring-Hertz 1992), but this was not observed during these investigations except when nematodes were present. During the density experiment those plates which received low fungal inoculum and were not induced to trap nematodes in the first week, were better colonised and trapped more nematodes after induction, than their counterparts that began trapping immediately. It seemed that trapping was occurring at the expense of hyphal extension growth. The results of this experiment confirmed this hypothesis.

Hyphal growth in the saprophytic colonies was aproximately double the rate of that in predaceous colonies. It could be argued that this was because the saprophytic colonies received more nutrient broth, but broth was added to compensate for the nutrients in the nematodes which were added to the predatory colonies. Given the limitations of this small-scale experiment, it does seem that there is a reduction in extension growth in colonies entering the predaceous phase.

The exsheathing of infective larvae in nutrient broth was an interesting phenomenon. This response was rapid and uniform among the many thousands of larvae added to the plates. Loss of the sheath did not prevent capture, as numerous larvae were subsequently trapped. This conflicts with the findings of Wharton and Murray (1990), who used weak hypochlorite to exsheath infective *Trichostrongylus colubriformis* larvae, and found they were not susceptible to the adhesive traps of *A. oligospora*. Possibly the J3 cuticle of *Heterorhabditis bacteriophora* differs from that of *T. colubriformis*, but when hypochlorite was used in an attempt to repeat their work during this project, traces

adhering to the larvae killed the fungus completely, and no nematodes were trapped.

The rate of hyphal extension could affect the ability of trapping fungi to colonise animal faeces. Clearly it is important for the fungus to occupy sufficient volume so that nematode larvae have a high probability of encountering traps during their sojourn in the faeces. Nematophagous fungi are generally considered (Cooke, 1962; Duddington, 1957; Mankau, 1962; Tribe 1980) to be poor competitors in their saprophytic phase, as is evident by the requirement for low nutrients and bait nematodes to detect their presence in soil samples. In the absence of nematodes, they may fail to establish in faeces, while the presence of nematodes allows them to establish but reduces their colony volume. The time between arrival of fungus in the faeces and emergence of larvae from faecal eggs could have some influence on both the extent of colonisation and amount of trapping effort exerted by a trapping fungus.

The results of these experiments suggest that the outcome of introducing nematophagous fungi to control nematode pests is sensitive to initial conditions, which may affect the volume of faeces colonised and the intensity of subsequent trapping. The decline in trapping effort found during the second and third weeks of the experiment, when 5,000 bait nematodes were added, resulted in higher nematode numbers at the end of the observation period than were found in plates which had received 50,000 bait nematodes. This may indicate that predatory fungi would be effective only during severe outbreaks of pest nematodes, while allowing potentially damaging numbers of them to escape predation under more normal circumstances.

The location of predatory fungi within the pasture system could affect their activity. In dense, moist pasture mats, trapping fungi may be able to colonise plant surfaces and trap infectives migrating from faeces. In these conditions, development of larvae would also be favoured and there may be a steady flow of infective larvae migrating to the pasture. While the fungus would trap many of these, many others would escape predation and

migrate higher up the grass, out of reach of the fungus. A rapid increase in nematode numbers could stimulate the fungus into greater activity and result in fewer reaching the higher grass than before. Under these circumstances, the fungus may prevent an epidemic but have little effect on chronic infestation.

Another possibility is that the increased nematode density occurring in the immediate vicinity of faeces during migration of infective larvae would be sufficient to stimulate greater trapping activity in predaceous fungal colonies. If this were the case, we could expect significant reductions in pasture infectivity to result from the introduction of the fungus, provided that the pasture mat maintained sufficient moisture for the inoculum to establish and survive. It seems far more likely, however, that sufficient moisture for fungus survival would be found within the faeces (Larsen, Thesis, 1991), and that the fungus must colonise that substrate in order to control nematode numbers.

Fresh faeces may be a more favourable environment for predatory fungi to colonise. Propagules entering faeces either from the soil, or some other form of inoculum, would be subject to competition from other microorganisms, but would have a competitive advantage once nematodes became available for trapping. If the faeces came from a severely parasitised animal, trapping activity could be intense, but in faeces with low faecal egg counts the fungus may have little or no effect.

There are also implications for the long-term effect of the introduction of predatory fungi on the survival of free-living soil nematodes. After the initial flurry of trapping activity has occurred in fresh faeces, predation is likely to decline. Subsequent arrivals of free-living nematodes will suffer little predation, allowing decomposition of the faeces to procede as usual. It is therefore unlikely that the introduction of a predatory fungus would have any long-term effect on non-target nematode species.

10.3. Formulation Tests.

The spore powder used in trials was of relatively low inoculum strength at 600,000 spores/g. Considering that 300 million spores could be contained in 1 ml of aqueous suspension and that much of the weight of this would be water, the great majority of the spore powder material must have been crushed spent grain. The powder used by Soprunov (1958) in his investigations contained 1.5 - 2 million conidia per gram. This too, must have been largely composed of spent grain. Soprunov reported negligible loss of viability after 16 months' dry storage at room temperature. This, together with the ease of its preparation, are valuable features of this form of fungal inoculum. However, the spent grain may be undesirable for inclusion in spore preparations if it offers a substrate for colonisation by fungal antagonists, or if it produces toxic fermentation products in the rumen, when administered to animals.

Spore powder was used in the attempts to protect fungus from rumen degradation by coating conidia in formalinised casein. This may have contributed to the failure of the preparation to survive extended periods in the Rusitec chamber, although it was the preparation procedure that killed the spores in the second encapsulation attempt. Ferguson and Solomon (1966) found that protective coatings must be free of minor imperfections such as pinholes to fully protect coated materials from rumen degradation. The spore powder contained some coarse fibrous particles which may have interfered with encapsulation. Further investigations may reveal that encapsulation in formalinised casein can provide the necessary protection for conidia if spent grain is excluded from the formulation.

Encapsulation in clay/alginate beads did not harm the fungus, and the procedure utilised conidia harvested in bentonite slurry, thus excluding spent grain and omitting the drying and milling required for the preparation of spore powder. The clay/alginate beads did not enable fungus to survive passage through the test sheep.

Bentonite slurry and canola oil both overcame the problems associated with hydrophobicity when harvesting conidia from solid cultures. Survival of spores in the slurry was high during the few weeks of observations, which was longer than any of the spray preparations were stored before their use in trials.

The sprays used in the fourth spray concentration trial and the timing trial were prepared from petri dish cultures by immersing the agar discs in water and mashing by hand in a plastic bag. This also resulted in good separation of the conidia from spent culture media, without any noticeable problems caused by hydrophobicity.

10.4. Survival of Fungus During Passage through Sheep

10.4.1. *In Vitro* Trials

Survival of the conidia of *Arthrobotrys oligospora* was limited to approximately four hours in the Rusitec, with most mortality occurring within two hours. This is consistent with the results of Larsen (Thesis, 1991), that conidia of *Arthrobotrys oligospora* survived up to four hours in diluted rumen fluid. Inhibition zones around rumen solids on agar plates indicated the presence of a toxin, and the removal of a strip of agar prevented the spread of the inhibiting agent, suggesting it was non-volatile. When rumen fluid was placed in wells cut into agar plate cultures of *A. oligospora*, no inhibition was observed. The toxin may be a product of fermentation, such as acetic or propionic acid. The concentration of volatile fatty acids in rumen fluid varies with the ingestion of food and its subsequent fermentation. The fluid taken from the Rusitec chamber and placed in wells cut in the agar in the test for inhibition by rumen fluid, was not examined for its volatile fatty acid content, and diffusion may have reduced concentrations to below toxic levels. When fermenting solids were placed on the agar surface, fermentation products could have reached higher concentrations in the surrounding agar than would normally be found in rumen fluid. The survival of fungal spores in the rumen may be enhanced if intimate contact with fermenting solids can be avoided.

10.4.2. *In Vivo* Trials

Baited Plates Sensitivity

Nematophagous fungi are relatively slow growing fungi, and this may be why it was three days before trapping fungus emerged from the mounds of faecal material on the test plates. After that time, the number of mounds from which trap-bearing hyphae emerged did not seem to be related to the number of conidia placed in them, although from some mounds there was no emergence of fungus. However, by the end of the observation period, 37 of the 40 faecal mounds had yielded a positive result. These results show an 80% probability of detecting a single viable propagule in a faecal sample on a baited plate, provided sufficient time is allowed for the fungus to emerge. From this it would seem that the inability to detect viable propagules in faecal samples was due to their absence, rather than a deficiency in the method of detection.

Preliminary Trials

Fungus preparations including a suspension of powdered grain cultures, conidia in canola oil emulsion, conidia pelletised in gel beads and in formalinised casein were delivered orally to the sheep but no viable propagules could be detected in the faeces until a whole grain millet culture was given. It seemed that the fungus had survived within the millet grains and viable propagules were being excreted with the faeces 42 hours after dosing. This result was the basis for conducting the larger scale trial in which the effects of grain size and culture age on fungus survival were examined.

The inability to detect viable predaceous fungus after administering other preparations was unexpected, considering the reports of Hashmi and Connan (1989) and Gruner et al. (1985). The immediate thought was that the method of detection was not sufficiently sensitive, but an investigation found that on baited plates, the presence of a single spore can be detected in faeces. While it remains possible that viable spores were being passed, but in insufficient numbers for any to have been included with the few grams of faeces spread on baited plates, such a poor survival rate would preclude biocontrol of nematode larvae.

Feeding Trials

The negative results of the grain culture feeding trials demonstrates that *A. oligospora* cannot be expected to survive when given in a feed supplement, unless it can be protected from degradation in the rumen. Hashmi and Connan (1989) stated that *A. oligospora* is commonly passed in the faeces of cattle, and Gruner *et al.* (1985) recovered *A. tortor* from lamb faeces after feeding millet grain cultures. However, when Hashmi and Connan gave 8 million conidia of *A. oligospora* daily, to calves in a field trial, they found only a small reduction in pasture infectivity. They concluded that only a small amount of the inoculum survived passage. The amount of millet fed to the lambs by Gruner *et al.* was much more than was given to sheep in these trials, being 100g/6 kg liveweight, which, for a three month old lamb, must have been a large proportion of its 300g daily concentrate allowance. The sudden introduction of so much millet to their diet could easily have altered the flow of digesta, hastening passage of the grain. Possibly the fungal species used were more resistant to degradation than was *A. oligospora.*

When viable fungus appeared in the faeces of the sheep used in the preliminary trials, it indicated that the results of Gruner *et al* were repeatable and that millet grains offered the degree of protection and speed of passage necessary for fungal survival. The importance of the method of delivery was overlooked as possible reason for the positive result.

From the results of the Rusitec trials, two important facts can be gleaned. First, the agent responsible for spore death was associated with the rumen solids. Second, spores exposed to a sub-lethal dose showed delayed germination. So the faecal samples taken after 24 and 42 hours may well have contained viable spores which were delayed in germinating, which would explain why no fungus appeared during the first week of observations. After three weeks, the numbers of positive replicates from the 18 hr, 24 hr and 42 hr samples were 6, 4 and 6 respectively, out of a possible 7. How could fungus have survived in this trial when similar material fed to sheep in the

feeding trials was unable to survive? It seems likely that the method of delivery made the vital difference.

In the preliminary trial, two tubs (each containing 80g) of millet grain culture were mixed with 400 ml water and given to the sheep as a drench, delivered from a glass bottle. Not only was this twice the quantity of grain administered in the second trial, but the method of delivery was very different. In the second trial, 80g of the grain culture was mixed with the sheep's usual ration of lucerne chaff and pellets. When ingested with the food ration, the grain would have been stored in the dorsal sac with the food bolus, as newly-ingested food is found packed into this part of the reticulo-rumen after a period of eating (Balch, 1961). The more fluid digesta are found in the reticulum, atrium ventriculi and the anterior part of the ventral sac. Newly-ingested solids in the dorsal sac are bathed in fluids during ruminal contractions, inoculating them with microorganisms to initiate fermentation. This would tend to result in the ingested fungal material being held in close contact with rumen solids during the hours following ingestion. On the other hand, grain delivered with water would go directly to the reticulum or join the fluid rumen contents, bypassing the dorsal sac and thus having a higher probability of escaping the rumen before degradation occurred.

Mertens (1990) used the term "escapability" rather than particle size to describe the kinetics of particles in the reticulo-rumen. Small particles in the rumenal mat pass differently to those in the reticular "zone of escape".

Mean retention time in the rumen for solids varies between particles of different size, but estimates of 35 hrs in a 30 kg sheep, and 47 hr in a 555 kg heifer (Van Soest, 1982), indicate that a tenfold extension of the survival time of the fungus would be a minimum requirement for its inclusion with a feed supplement. The same source gives the mean retention time of liquids as 19 hr and 15 hrs respectively, suggesting that even when delivered in liquid form, the fungal spores would require protection from degradation to achieve high rates of survival. The

retention time in the rumen is roughly half the time required for passage through the whole digestive tract, this being 70 hrs for particles and 38 hours for liquids in a 30 kg sheep (Van Soest, 1982). Therefore, even though millet seeds appeared in the faeces collected 18 hours after dosing (preliminary investigation), this must have represented only a small proportion of the total dose, with the major part arriving in the faeces collected in the 42 hour sample. It appears that germination of viable spores included in the later faecal samples was delayed for at least a week. This is in keeping with the results of the Rusitec trial, in which fungus immersed for periods of three or four hours showed delayed germination of five and seven days respectively.

It was noted that after ten days in a plastic bag on the laboratory bench, infective larvae had emerged from faeces in quantities sufficient to be seen by the unaided eye. This is consistent with the minimum times found by Callinan (1978a, 1978b, 1979) for the appearance of L3 larvae on herbage. The results of the timing trial also indicate that no significant reduction in infective larvae can be expected if the fungus arrives in the faeces seven days after deposition. The reduction in pasture contamination by the inclusion in faeces of a fungus suffering such delayed germination could be expected to be minimal.

The survival of fungal spores in millet culture delivered in a liquid drench offers some grounds for optimism. Clearly, the fate of *A. oligospora* ingested with solid food is death, since no survival was found during the feeding trials. When fungal spores are ingested with solid food, sequestration in the dorsal sac brings them into intimate contact with fermenting solids for hours before they enter the fluid contents of the rumen and have a chance of passing to the omasum. Some form of protective coating may prevent the death of spores in the rumen, but for rapid germination to occur when faeces are voided, it would be necessary for the coating to be removed during further passage through the digestive tract, and for the fungus to capable of survival during this passage.

Conidia of *A. oligospora* survived at pH 2.2 for six hours, which is longer than the mean residence time of 0.5-1.0 hour reported by Hyden (1961). Abomasal pH is rarely below 2.3-2.4 (Comline and Titchen, 1961). Polymers soluble at this pH, but insoluble at rumen pH (6.5-7.0) have been developed (Ferguson and Solomon 1966; Farbenfab. Bayer, 1966; Grant and Mench, 1969; Miller, 1972, 1973) and may protect fungal spores within the rumen; however, they could not be obtained for use in this project. Analysis of the results of the preliminary trial suggest that *A. oligospora* can survive passage, if it is viable when it is past the rumen.

The transit times for fluids in the abomasum, small intestine, caecum and colon-rectum of a sheep were given by Hyden (1961) as:

Abomasum	0.5 - 1	hours
Small intestine	1 - 2	"
Caecum	6 - 11	"
Colon-Rectum	10 - 11	"

This gives a range of 17.5 - 25 hours for total retention time post-rumen, which is in agreement with Van Soest's (1982) estimated mean of 19 hours. Clearly, fungus voided in faeces 18 hours after dosing would have spent very little time in the rumen. At 24 hours after dosing, most of the fungal spores voided in the faeces would have spent less than four hours in the rumen. It must be remembered that faeces were collected in a calico bag, and the 24 hour sample was, in fact, defecated between 18 and 24 hours after dosing. Similarly, the 42 hour sample was really a 24 - 42 hour sample. The retention times given above refer to fluids, whereas the millet grains and fungal spores are particles, which would tend to have longer retention times than the above estimates. The chances of fungus which exited the rumen in less than four hours being present in the 42 hour sample would be very high. The results obtained in the preliminary millet trial are consistent with the hypothesis that the fungal spores were able to survive passage once past the rumen.

Waller *et al.* (1994) recovered *A. oligospora* from a faecal sample taken 24 hours after oral dosing. The dose given was 1.2 million conidia in four gelatin capsules containing conidia in 1 ml of water, given at hourly intervals by stomach tube. Samples were taken from the abomasum by catheter at hourly intervals up to 12 hours, and at 12 hourly intervals afterwards. Samples were incubated at 25°C in vermiculite cultures baited with 15,000 *H. contortus* larvae initially and again after 7 days. Cultures were examined after 14 days for the presence of trapping fungi, and remaining nematodes counted. The last appearance of *A. oligospora* in the abomasum was in the 8 hour sample, four hours after the last capsule was administered. Conidia escaped the rumen rapidly, being detected in the abomasum one hour after the first dose. These results are consistent with the survival of *A. oligospora* conidia being limited to 4 hours in the rumen.

When conidia were placed in the abomasum, the only time viable spores were collected in the faeces was 9 hours after dosing. This is indicative of a much more rapid rate of passage than would be expected, considering the findings of Hyden (1961). After that time, *A. oligospora* was not detected, indicating that this fungus has very poor survival post rumen. Unfortunately, the species of fungi selected from faecal samples (Larsen *et al.* 1994) were not included in these trials. All fungi used in these trials came from a European collection, and it is quite possible that native Australian species would differ in both their predacity and gut survival potential.

10.5. Glasshouse Trials

The first glasshouse trial confirmed the ability of *Arthrobotrys oligospora* to reduce the numbers of larvae surviving to infective stage when applied as a spray to whole faeces lying on a pasture sward. It also showed that the fungus must be placed in contact with the faeces to achieve its effect, rather than relying on colonisation of faeces by soil-borne fungal inoculum. The majority of infective larvae had migrated to the pasture within three weeks, and even if soil-borne fungi had colonised the faeces by this time, it would have been too late to prevent pasture contamination. Young (1983) found that both *Ostertagia ostertagi*

and *O. circumcincta* migrated to pasture immediately after developing to L3 stage, given sufficient moisture, and maximum yields on herbage occurred after 33 days for *O. circumcincta* and *Trichostrongylus axei* (Callinan, 1977, 1978), and 38 days for *T. vitrinus* (Callinan, 1979) under field conditions in Western Victoria. Mean development times were reported as from 12 to 14 days. Between 60 and 75% of the L3 population was found on the herbage with less than 8% found in the soil during these investigations. Nematophagous fungi are frequently isolated from old manure (Duddington, 1957; Barron 1977; Hashmi & Connan, 1989) and may well enter manure from the soil given time, but the result of this trial suggests that soil-borne trapping fungi may have little effect on pre-parasitic larval survival. This may not be the case for some species of parasitic nematodes, such as *Trichostrongylus vitrinus*, in which the L3 larvae may persist in the faeces for lengthy periods (Callinan, 1979).

It was shown that *A. oligospora* became established in the pasture tubs. Mycelia were clearly visible as the grass was growing, and all ten randomly selected soil samples, taken the week before the trial commenced, produced trapping fungus on baited plates. By this time a pasture mat had established, and this would have remained moist most of the time, as the tubs were regularly watered. Temperatures during the trial period reached 30°C during the day, and fell to about 15°C at night (see appendix for details). If colonisation of the pasture mat occurred (as discussed in section 2), this had little effect on migrating nematodes or colonisation of faeces by soil-borne fungi. The high temperatures recorded during the trial may have affected the ability of fungus to enter faeces from the soil. In less extreme conditions, and in a deeper pasture sward (the grass was cut to 2 cm at commencement and at weekly intervals during the trial), perhaps the fungus would colonise faeces more rapidly from the soil. However, under the conditions of this trial, this did not occur.

The reduction obtained by spraying ranged from about 70% to 90% of the infective larvae recovered from control plots. This compares favourably with the reductions reported by Hashmi and Connan (1989) of 72% and Grønvold (1987) of 86% and 90%.

The spray used in this trial was assayed at 34,000 conidia/ml. This was considered to be a fairly dense inoculum, and it was hoped that spray concentration trials would show that similar results could be obtained using lower inoculum rates.

Spray Concentration Trials

The first concentration trial seemed to show a 30% reduction in infective larvae at the highest rate (of 1,500 spores/ml), but this was offset by an increase in the numbers of infective larvae from plots sprayed at the rate of 750 spores/ml. Analysis of variance showed that treatment differences were not significant at the $P<0.1$ level. In fact, nematode survival was generally poor in this trial, with fewer than 3,000 infective larvae recovered from each plot. At 1,800 eggs/g, 35g of faeces could have produced 63,000 infective larvae per plot, so the survival rate was less than 5%. It is not surprising, considering the variance (in survival) that could be expected between plots, that the effect of spraying was insignificant. It is also possible that prey density was too low to have induced trapping in the fungi.

Results of the second trial were also disappointing. Fifty grams of faeces containing 1,100 epg could have yielded 55,000 infective larvae per plot, but the average recovered over all plots was 2,000, a survival rate of less than 4%. Once again, fungus effects were not statistically significant. However, Callinan (1978a, 1978b, 1979) found maximum yields of 16%, 5.05% and 8.9% from faecal egg numbers of *O. circumcincta, T. vitrinus* and *T. axei* in field experiments, so the yields of infective larvae from the glasshouse plots were similar to those which could be expected under field conditions.

In the third spray trial, 60g faeces containing 1,000 epg was placed on each plot. Survival in the control plots averaged 18%, but this was reduced to 6.6% in plots sprayed with 10,000 spores/ml. This time differences were significant at the $P<0.05$ level. However, the second highest spray treatment of 1,000 conidia/ml had an average infective larval count greater than the controls, with a survival rate of 25% This was partly caused by one of the treatment replicates having the highest number of

nematodes of any plot, which was double the average number in controls. Even if this were disregarded, the reduction in numbers attributable to the fungus was only 64%, which was less than that obtained in the first glasshouse trial. It seemed obvious that even higher concentrations of fungus would be needed.

Higher concentrations were used in the fourth spray trial, but reductions in nematode numbers still only reached about 66%. Analysis showed that these results were significant at the $P<0.05\%$ level, with survival rates averaging 10% in the controls, and 3% at the highest spray rate. The need for the high concentrations of fungus indicated by fig. 5.2.6., was a surprise, considering the results of Waller and Faedo (1993). In their experiments with *A. oligospora,* significant reductions in larval yields were obtained from concentrations as low as 10 conidia/g faeces, and optimum capture rates observed at both 100 and 250 conidia/g. Perhaps conidia were washed off the faeces during spraying. A microscopic examination of the highest dose spray suspension revealed a very low proportion of volume occupied by conidia. Possibly, the use of a thickener would increase the number of conidia retained on a pellet, and thus make lower concentrations more effective. A thickener may also cause spores to remain on the grass sward for longer, and extend the duration of effect obtainable from a single spray application.

Timing

The timing trial examined the effect of applying the fungus at intervals both before and after the faeces were deposited on the pasture. The ability of the fungus to grow in faeces was demonstrated in the laboratory when spores mixed with fresh faeces spread on baited water agar plates produced traps within 24 hours. However, in practise, the fungus may encounter faeces which have been on the ground for several days, by which time microbial competition may impair colonisation by the fungus. Development of pre-parasitic nematodes may also be well on the way after seven days, enabling the majority of larvae to migrate before the fungus can form large trapping networks. The results of the timing trial suggest that this was the case, with great variability in the outcomes of plots sprayed three days after

deposition of faeces and no reduction in the infectivity of plots sprayed one week after faeces addition. Also, there was little residual effect three days after spraying, and no effect one week after spraying.

Mean development times from egg to L3 may be 18-20 days (Croll and Matthews, 1977), but minimum times for appearance of L3 on herbage may be much shorter than this. Callinan reported times of 4-27 days for *O. circumcincta* (1978a) with a mean minimum of 14.3 days, and 4-28 days for *T. axei* (1978b) with a mean minimum of 12.3 days. Considering these observations, it is not surprising that no reduction was found in plots sprayed 14 days after the placement of faeces.

The residual effect of the spray was not clear. Only two of the five plots sprayed three days before the faeces were deposited developed normal numbers of infective larvae. Also, one of the seven day replicates yielded very low numbers of infective larvae, suggesting that spores may have remained on the herbage long enough to enable the fungus to germinate in the faeces deposited on this plot. The trial was conducted in a glasshouse, and watering was suspended during this period, but on one occasion, the day after spraying, an attendant ignored the "do not water" signs, and watered all the pasture tubs. Since the tubs were randomised, some tubs to which faeces had not yet been added may have received more water, or may have been more vigorously hosed, than others. Fungal spores washed from the grass into the soil, would have had little effect on developing larvae. The results suggest more trials would be warranted to determine the effect of rain following spraying, and to determine the duration of fungal effectiveness in the absence of rain.

These results indicate that spraying would need to be a daily treatment to achieve consistent reductions in pasture infectivity. Thus spraying would appear to be impractical for controlling parasitic worms. As a means of introducing a fungal species which could maintain itself in the pasture environment (such as by surviving passage through sheep), spraying could be useful,

but inoculum would be most effective when applied to fresh faeces.

Considerable progress has been made towards finding a more suitable fungus by workers at the CSIRO McMaster laboratories. After screening a large number of faecal samples for the presence of trapping fungi, Larsen *et al.* (1994) found several isolates of *Arthrobotrys oligospora* and *Duddingtonia flagrans* which were capable of surviving gut passage through sheep. Work is now under way (Waller *pers comm*) to find a practical method for administration of inoculum to sheep.

A further obstacle to the usefulness of *Arthrobotrys oligospora* is the high concentration of spores needed to achieve significant reductions in infective larvae. The rate of one million spores per millilitre, used in the fourth spray trial, reduced survival to about thirty percent of the controls. The required rate to get 90% reduction would be 100 million spores per ml, by extrapolation of existing data. Early trials were hampered by poor overall survival of the worms, characterised by low numbers of nematodes recovered in all treatments, masking any effect the fungus may have had. The downward trend in the second trial results and, without the anomalous 1,000 spores/ml result, the trend in the third trial result, are very similar to that of the fourth trial, and are all indicative of fungal suppression of viable nematodes.

Timing influenced the efficacy of spraying, and possibly the variable age of faeces used in previous experiments contributed to variance within treatments. Spraying is probably not the best method for delivering fungus (a species which could survive in the digestive tract may be given as a feed additive or a lick), but spraying offers a method by which predaceous fungi could be employed by farmers using commonly available equipment.

CONCLUSION

Integrated control of animal-parasitic nematodes involving pasture management, animal breeding and biological control in addition to conventional methods, appears to be the future approach to parasite control. Results obtained during this project suggest that it should be possible to include biological control in the strategy, and indicate ways in which it might be achieved. The effect of a fungal agent if successful would be to depress the numbers of infective larvae on pasture herbage to below the level which causes production losses in grazing livestock.

If the level of intake of infective larvae can remain low, livestock show no ill effects (Symons, 1989) and benefit from the immunity to parasitism that develops as a result of exposure. However, even in immune animals, production losses can result from exposure to high levels of infective larvae (Armour, 1994).

The farm soil survey showed that nematophagous fungi were not common in the surface layer of pasture soils. There does not appear to be any existing association between a predatory fungus and the gastro-intestinal parasites of ruminants. If a fungus, capable of forming a predator/prey relationship can be introduced, it is likely to be of some benefit to livestock production.

Introducing predaceous fungi by soil inoculation will have little or no effect on parasite ecology. The glasshouse trials showed that soil-borne fungus was incapable of producing significant reductions in nematode numbers on herbage. Spores must be placed directly in contact with contaminated faeces for the fungus to be effective.

Spraying spore suspensions onto contaminated faeces can reduce nematode survival by up to 90%, but the faeces must be fresh for the spray to be effective. There is some residual effect on faeces deposited after pastures have been sprayed, but this lasts for only a few days. Possibly the use of a thickening agent in the spray would extend the residual effect.

The concentration of spores required for the spraying of pasture to be effective was considered too high for practical purposes; if large areas were to be sprayed, the amount of fungal material needed would be enormous. This requirement could possibly be reduced by the use of a thickening agent to prevent loss of spores in spray runoff.

Of the nematophagous fungi tested, *Arthrobotrys oligospora* was the fastest growing and most predaceously active. It was also easily produced in relatively large quantities, withstood drying and could be stored wet or dry. Although it meets many of the criteria for a successful biocontrol agent, it did not survive when fed to sheep and could not be expected to be self-sustaining in a pasture system.

Destruction of fungal spores occurs in the rumen and is associated with fermenting solids. Placement of fungus in the animals' feed causes inoculum to be held in intimate contact with fermenting solids for several hours after ingestion, destroying fungal viability. When a fungal complex is delivered in water, it facilitates early escape from the rumen and thus survival of the fungal material through to the faeces.

There have been a number of patents registered for the protection of substances within the rumen (see Chapula, 1975, for review). Possibly *Arthrobotrys oligospora* spores could be protected from rumen degradation and the fungus could be given as a feed additive. There would be no point in developing this however, if other species of fungus can survive without protection.

There may be some livestock producers who would adopt spraying to control worms even though it is far from ideal. If spraying could be effective on a weekly basis, it could be used in highly contaminated areas such as holding paddocks on dairy farms (particularly goat dairies) and by hobbyfarmers who run livestock on the same ground continuously. These operators tend to have chronic parasite problems which they presently treat by

frequent anthelmintic use. The onset of anthelmintic resistance is inevitable in such operations but may be delayed or prevented if an alternative such as biocontrol were available.

REFERENCES

ABBOTT, K.A. & McFARLAND, I.J. (1991) "*Trichostrongylus axei* infection as a cause of deaths and loss of weight in sheep" *Aust. Vet. J.* 68(11): 368-369.

ABU-LABAN, A.Z. & SALEH, H.M. (1992) "Evaluation of animal manures for mass-production, storage and application of some nematode egg parasitic fungi." *Nematologica* 38(2): 237-244.

ANDERSON, K.A.; MARTIN, P.J. & JARRETT, R.G. (1991) "The efficacy of mixtures of albendazole sulphoxide and levamisole against sheep nematodes resistant to benzimidazole and levamisole" *Aust. Vet. J.* 68: 127-132.

ANDERSON, R.M.; MARTIN, P.J. & JARRETT, R.G. (1991) "Field evaluation of a mixture of albendazole sulphoxide and levamisole against *Ostertagia* and *Trichostrongylus* spp in sheep" *Aust. Vet J.* 68: 133-136.

ANDERSON, R.M. & MAY, R.M. (1979) "Population biology of infectious diseases: Part I" *Nature* 280: 361-367.

ANDERSON, R.M. & MAY, R.M. (1979) "Population biology of infectious diseases: Part II" *Nature* 280: 455-461.

ANDERSON, R.M.; MAY, R.M. & GUPTA, S. (1989) "Non-linear phenomena in host-parasite interactions" *Parasitology* 99: S59-S79.

ANDERSON, N. & WALLER, P.J. *Resistance in Nematodes to Anthelmintic Drugs* Aust. Wool Corp. Technical Publication, CSIRO, Aust. 1985.

ANONYMOUS, 1971 "How to protect protein" Page 22 in *Rural Research in CSIRO*, June.

ARMOUR, J. (1994) "Veterinary helminthology 1954-1993: a personal view of four decades of research" *Vet. Parasitol.* 54: 11-22.

ARMOUR, J. & BRUCE, R.G. (1974) "The inhibition of development of *Ostertagia ostertagi* - a diapause phenomenon in a nematode." *Parasitology* 69: 161-174.

BAILEY, F. & GRAY, N.F. (1989) "The comparison of isolation techniques for nematophagous fungi from soil" *Ann Appl Biol.* 114: 125-132.

BAILEY, F. & GRAY, N.F. (1989) "The effect of replication on the probability of isolating nematophagous fungi from soil" *Plant Soil* 113(1): 141-143.

BALAN, J. & GERBER, N. (1972) "Attraction and killing of the nematode *Panagrellus redivivus* by the predaceous fungus *Arthrobotrys dactyloides*." *Nematologica* 18: 163-173.

BALCH, C.C. "Movement of Digesta through the Digestive Tract" Ch. 3 in *Digestive Physiology and Nutrition of the Ruminant* (D. Lewis Ed.) Butterworths, Lndn. 1961.

BALDOCK, F.C.; LYNDAL-MURPHY, M. & PEARSE, B. (1990) "An assessment of a composite sampling method for counting strongyle eggs in sheep faeces" *Aust. Vet. J.* 67(5): 165-167.

BARGER, I.A.; HALL, E. & DASH, K.M. (1991) "Local eradication of *Haemonchus contortus* using closantel" *Aust. Vet. J.* 68(10): 347-348.

BARGER, I.A.; STEEL J.W. and RODDEN, B.R. (1992) "Effects of a controlled-release albendazole capsule on parasitism and production from grazing Merino ewes and lambs" *Aust Vet J.* 70(2): 41-48.

BARNES, E.H.; DOBSON, D.J.; DONALD, A.D. & WALLER, P.J. (1988) "Predicting populations of *Trichostrongylus colubriformis* infective larvae on pasture from meteorological data" *Int. J. Parasitol.* 18(6): 767-774.

BARRON, G.L. "*The Nematode-Destroying Fungi* " Can. Biol. Pubs. Ontario, Canada. 1977.

BARRON, G.L. (1978) "Nematophagous Fungi: Endoparasites of *Rhabditis terricola* " *Microb. Ecol.* 4: 157-163.

BARRON, G.L. & THORN, R.G. (1987) "Destruction of nematodes by species of *Pleurotus*" *Can. J. Bot.* 65: 774-778.

BELL, S.L.; THOMAS, R.J. & FERBER, M.T. (1988) "Feed intake of grazing calves exposed to trichostrongyle infection and treated

with the morantel sustained release bolus" *Vet. Parasitol.* 28(1/2): 125-136.

BERNARD, E.C. & ARROYO, T.L. (1990) "Development, distribution, and host studies of the fungus *Macrobiotophthora vermicola* (Entomophthorales)" *J. Nematol.* 22(1): 39-44.

BIRD, A.F. *The Structure of Nematodes* Academic Press. N.Y., London. 1971.

BLACKBURN, F. & HAYES, W.A. (1965) "Studies on the nutrition of *Arthrobotrys oligospora* Fres. and *A. robusta* Dudd. I.The saprophytic phase" *Ann. Appl. Biol.* 58: 43-50.

BOAG, B. & LOPEZ-LLORCA, L.V. (1989) "Nematodes and nematophagous fungi associated with cereal field and permanent pasture in Eastern Scotland" *Crop Res.* 29(1): 1-10.

BOAG, B.; ROBERTSON, W.M.; AINSWORTH, L.F. (1988)"Observations on the specificity of the nematophagous fungus *Arthrobotrys dasguptae* (Shome & Shome) to plant-parasitic nematodes." *Nematologica* 34: 238-245.

BOWN, M.D.; POPPI, D.P. & SYKES, A.R. (1991) "The effect of post-ruminal infusion of protein or energy on the pathophysiology of *Trichostrongylus colubriformis* infection and body composition in lambs" *Aust. J. Agric. Res.* 42: 253-67.

BOYETTE, C.D.; QUIMBY, P.C.; BRYSON, C.T.; EDGLEY, G.H.; FULGHAM, F.E. (1993) "Biological control of hemp sesbania (*Sesbania exaltata*) under field conditions with *Colletotrichum truncatum* formulated in an invert emulsion" *Weed Science* 41(3): 497-500.

BRUNSDON, R.V. (1980) In *Ostertagia Symposium "80"* Roseworthy Agricultural College, Roseworthy, South Aust. May 1980 p.79.

BRYAN, R.P. & KERR, J.D. (1989) "Factors affecting the survival and migration of the free-living larvae of gastrointestinal nematode parasites of cattle in central Queensland" *Vet. Parasitol.* 30(4): 315-326.

BRYAN, R.P. & KERR, J.D. (1989) "The relation between the natural worm burden of steers and the faecal egg count differentiated to species" *Vet. Parasitol.* 30(4): 327-334.

CALLINAN, A.P.L. (1978a) "The ecology of the free-living stages of *Ostertagia circumcincta* " *Int. J. for Parasitol.* 8: 233-237.

CALLINAN, A.P.L. (1978b) "The ecology of the free-living stages of *Trichostrongylus axei* " *Int. J. for Parasitol.* 8: 453-456.

CALLINAN, A.P.L. (1979) "The ecology of the free-living stages of *Trichostrongylus vitrinus*" *Int. J. for Parasitol.* 9: 133-136.

CALLINAN, A.P.L. & MORLEY, F.H.W. (1982) "Control of nematode parasites in sheep" *Proc.of the Aust. Soc. of Anim. Prod.* 14: 43-44.

CALLINAN, A.P.L. & WESTCOTT, J.M. (1986) "Vertical distribution of Trichostrongylid larvae on herbage and in soil" *Int. J. for Parasitol.* 16(3): 241-244.

CAYROL, J.C. (1980) "A fungus biocontrol for root-knot nematode" *International Potato Centre Circular* VIII(10).

CAYROL, J.C.; BRUN, J. (1975) "Study of the Predatory Ability of some Nematode-Trapping Fungi against the nematode *Caenorhabditis elegans* at Different Temperatures." *Rev. Zool. Agric. Pathol. Veg.* 74(4): 139-146.

CAYROL, J.C.; DJIAN, C. & PIJAROWSKI, L. (1989) "Study of the nematicidal properties of the culture filtrate of the nematophagous fungus *Paecilomyces lilacinus*" *Rev. Nematol.* 12(4): 331-336.

CAYROL, J.C.; FRANKOWSKI, J.P.; LANZA, R.; TAMONTE, M. (1991) "Nematodes in kiwifruit culture. Biological control trials with the nematophagous fungus T-350" *Revue-Horticole* 313: 54-56.

CHAPULA, W. (1975) *J. Dairy Sci.* 58: 1198.

COLE, V.G. *Animal Health in Australia* Vol. 8. *Helminth Parasites of Sheep and Cattle* Aust. Govt. Pub. Service, Canberra 1986.

COLES, G.C.; TRITSCHLER II, J.P.; GIORDANO, D.J.; LASTE, N.J. & SCHMIDT, A.L. (1988) "Larval development test for detection of anthelmintic resistant nematodes" *Res. in Vet. Sci.* 45: 50-53.

COMLINE, R.S. & TITCHEN, D.A. "Nervous Control of the Ruminant Stomach" Ch. 2 in *Digestive Physiology and Nutrition of the Ruminant* (D. Lewis Ed.) Butterworths, Lndn. 1961.

CONNICK, W.J.; BOYETTE, C.D.; McALPINE, J.R. (1991) "Formulation of mycoherbicides using a pasta-like process." *Biological Control* 1(4): 281-287.

CONNICK, W.J.; DAIGLE, D.J.; QUIMBY, P.C. (1991) "An improved invert emulsion with high water retention for mycoherbicide delivery." *Weed Technology* 5(2): 442-444.

COOKE, R.C. (1962) "The Ecology of Nematode-Trapping Fungi in the Soil" *Ann. Appl. Biol.* 50: 507-513.

COOKE, R.C. (1963a) "The Predaceous Activity of Nematode-Trapping Fungi added to Soil" *Ann. Appl. Biol.* 51: 295-299.

COOKE, R.C. (1963b) "Ecological Characteristics of Nematode-Trapping Hyphomycetes. I. Preliminary Studies" *Ann. Appl. Biol.* 52: 431-437.

COOKE, R.C. (1964) " Ecological Characteristics of Nematode-Trapping Hyphomycetes. II. Germination of Conidia in Soil" *Ann. Appl. Biol.* 54: 375-379.

COOKE, R.C. & GODFREY, B.E.S. (1964) "A Key to the Nematode-Destroying Fungi" *Trans. Brit. Mycol. Soc.* 47(1): 61-74.

CROLL, N.A. *The Behaviour of Nematodes* Edward Arnold Ltd, London. 1970.

CROLL, N.A. & MATTHEWS, B.E. *Biology of Nematodes* Blackie, Glasgow and London. 1977.

CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1977) "Design and development of a long-term rumen simulation technique (Rusitec)" *Br. J. Nutr.* 38: 371-384.

CZERKAWSKI, J.W. & BRECKENRIDGE, G. (1979) "Experiments with the long-term rumen simulation technique (Rusitec); use of soluble food and an inert solid matrix" *Br. J. of Nutr.* 42: 229-245.

DACKMAN, C. & NORDBRING-HERTZ, B. (1992) "Conidial traps - a new survival structure of the nematode-trapping fungus *Arthrobotrys oligospora*" *Mycol. Res.* 96(3): 194-198.

DACKMAN, C.; OLSSON, S.; JANSSON, H-B.; LUNDGREN, B.; NORDBRING-HERTZ, B. (1987) "Quantification of Predatory and

Endoparasitic Nematophagous Fungi in Soil." *Microbiol. Ecol.* 13(1): 89-93.

DAIGLE, D.J. & COTTY, P.J. (1992) "Production of conidia of *Alternaria cassiae* with alginate pellets." *Biological Control* 2(4): 278-281.

DAVIES, K.G. & DANKS, C. (1992) "Interspecific differences in the nematode surface coat between *Meloidogyne incognita* and *M. arenaria* related to the adhesion of the bacterium *Pasteuria penetrans*" *Parasitology* 105: 475-480.

DAVIES, K.G.; KERRY, B.R.; FLYNN, C.A. (1988) "Observations on the Pathogenicity of *Pasturia penetrans,* a parasite of root-knot nematodes." *Ann. Appl. Biol.* 112: 491-501.

DEACON, J.W. (1991) "Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens" *Biocontrol Science and Technology* 1: 5-20.

DESCAZEAUX, J. (1939a) "Stérilisation biologique du crottin parasité par des larves de Nématodes" *Bull. Acad. Vét. France* 12: 136-139.

DESCAZEAUX, J. (1939b) "Action des champignons Hyphomycètes prédateurs sur les larves de certains nématodes parasites des ruminants" *Bull. Soc. Path. exot.* 32: 457-459.

DESCAZEAUX, J. & CAPELLE, (1939) "Contribution à l'étude des champignons prédateurs de larves de nématodes parasites des animaux domestiques" *Bull. Acad vet. France* 12: 284-288.

DESCHIENS, R. (1939a) "Capture et destruction de larves de *Strongyloides* du singe et du boeuf par des Hyphomycètes" *Bull. Soc. Path. exot.* 32: 394-398.

DESCHIENS, R. (1939b) "Considérations relatives à la destruction des larves de nématodes parasites par des Hyphomycètes prédateurs." *Bull. Soc. Path. exot.* 32: 459-464.

DESCHIENS, R. (1939c) "Conditions de capture des larves de Dictyocaules par des Hyphomycètes prédateurs." *Bull. Soc. Path. exot.* 32: 698-700.

DESCHIENS, R. (1941) "Innocuité des Hyphomycètes prédateurs de nématodes pour la végétation des pâturage et pour le bétail." *C.R. Soc. Biol. Paris* 135: 830-832.

DESCHIENS, R. (1942) "Milieux de culture à rendement élevé pour la récolte des spores d'Hyphomycètes prédateurs de nématodes." *Bull. Soc. Path. exot.* 35: 237-241.

DESCHIENS, R. (1943) "La prophylaxie des infestations vermineuses à nématodes par les agents biologiques." *La Presse médicale, Paris* 16 *Oct* 2, no 39: 573-574.

DESCHIENS, R. & LAMY, L. (1943) "Conditions pratiques de culture, de sporulation et de récolte des spores d'Hyphomycètes prédateurs de nématodes." *C.R. Soc. Biol. Paris* 137: 381-383.

DOMSCH, K.H.; GAMS, W. & ANDERSON, T. H. (1980) "*Arthrobotrys* Corda 1839." In: *Compendium of Soil Fungi,* vol.1. pp. 59-64. Academic Press.

DONALD, A.D. (1967) "Population studies on the infective stage of some nematode parasites of sheep. I. The frequency distribution of strongyloide infective larvae in random samples of pasture." *Parasitology* 57: 263-279.

DONALD, A.D. (1968) "Ecology of the free-living stages of nematode parasites of sheep" *Aust. Vet. J.* 44: 139.

DONALD, A.D. (1994) "Parasites, animal production and sustainable development" *Vet. Parasitol.* 54: 27-47.

DONALD, A.D. & WALLER, P.J. "Problems and Prospects in the Control of Helminthiasis in Sheep" Ch.8. in *Biology and Control of Endoparasites* edited by Symons, Donald and Dineen, Academic Press, 1982.

DRESCHLER, C. (1933) "Morphological diversity among fungi capturing and destroying nematodes." *J. Wash. Acad. Sci.* 23: 138-141.

DRESCHLER, C. (1933a) "Morphological features of some more fungi that capture and kill nematodes."*J. Wash. Acad. Sci.* 23: 267-270.

DUDDINGTON, C.L. "*The Friendly Fungi*" Faber & Faber, London. 1957.

EREN, J. & PRAMER, D. (1964) "Carbon Dioxide-dependent Morphogenesis in *Arthrobotrys conoides*" *Nature* 204: 804.

FARBENFAB. BAYER, A.G. (1966) "Protective coating for therapeutic agents: Coated compositions and processes for the production thereof" German Patent 31,696.

FELL, L.R.; LYNCH, J.J.; ADAMS, D.B.; HINCH, G.N.; MUNRO, R.K. & DAVIES, H.I. (1991) "Behavioural and physiological effects in sheep of a chronic stressor and a parasite challenge" *Aust. J. Agric. Res.* 42: 1335-1346.

FERGUSON, K.A. & SOLOMON, D.H. (1966) "Methods and Food Composition for Feeding Ruminants" CSIRO Patent. Aust. Patent No. 402,712.

FLEMING, M.W. (1988) "Size of inoculum dose regulates in part worm burdens, fecundity and lengths in ovine *Haemonchus contortus* infections" *The J. of Parasitology* 74(6): 975-978.

FRAVEL, D.R.; MAROIS, J.J.; LUMSDEN, R.D. & CONNICK Jr., W.J. (1985) "Encapsulation of potential biocontrol agents in an alginate-clay matrix" *Phytopathology* 75: 774-777.

FRESENIUS, G. (1852). *Beiträge zur Mykologie.* Heft 1-2. pp. 1-80.

FRISCH, J.E. & HUNTER, R.A. (1990) "Interaction of Compudose 200 and resistance to parasites on growth of steers of two cattle breeds" *J. of Agric. Sci. Camb.* 115: 259-264.

FOWLER, M. (1970) "New Zealand predacious fungi" *N.Z. J. Bot.* 8: 283-302.

GAMS, W. (1988) "A contribution to the knowledge of nematophagous species of *Verticillium.*" *Netherlands J. of Plant Pathology,* 94: 123-148.

GIBSON, T.E. & EVERETT, G. (1967) "The ecology of the free-living stages of *Trichostrongylus colubriformis*" *Parasitology* 57: 533-547.

GIBSON, T.E. & EVERETT, G. (1972) "The ecology of the free-living stages of *Ostertagia ostertagi*" *Parasitology* 64: 451-460.

GIUMA, A.Y. & COOKE, R.C. (1971) "Nematoxin production by *Nematoctonus haptocladus* and *N. concurrens* " *Trans. Brit. mycol Soc.* 56: 89-94.

GIUMA, A.Y.; HACKET, A.M., & COOKE, R.C. (1973) "Thermostable nematotoxins produced by the germinating conidia of some endozoic fungi." *Trans. Brit. mycol Soc.* 60: 49-56.

GOMES-CARNEIRO, R.M.D. & CAYROL, J.C. (1991) "Relationship between inoculum density of the nematophagous fungus *Paecilomyces lilacinus* and control of *Meloidogyne arenaria* on tomato." *Revue de Nematologie* 14(4): 629-634.

GORDON, H.McL. (1948) "The epidemiology of parasitic diseases, with special reference to studies with nematode parasites of sheep." *The Aust. Vet. J.* 24: 17-45.

GRANT, P.M. & MENCH, J.W. (1969) "Orally applicable mass stable against breakdown in the ruminant paunch" Deutches Patentamt 1,945,650.

GRAY, N.F. & BAILEY, F. (1985) "Ecology of Nematophagous Fungi; Vertical Distribution in a Deciduous Woodland." *Plant and Soil.* 86: 217-224.

GRAY, N.F. (1984) "The Effect of Fungal Parasitism and Predation on the Population Dynamics of Nematodes in the Activated Sludge Process." *Ann. Appl. Biol.* 104(1): 143-150.

GRØNVOLD, J. (1989) "Induction of nematode-trapping organs in the predacious fungus *Arthrobotrys oligospora* (Hyphomycetales) by infective larvae of *Ostertagia ostertagi* (Trichostrongylidae)." *Acta Vet. Scand.* 30(1): 77-81.

GRØNVOLD, J.; HENRIKSEN, S.A.; NANSEN, P.; WOLSTRUP, J. & THYLIN, J. (1989) "Attempts to control infection with *Ostertagia ostertagi* (Trichostrongylidae) in grazing calves by adding mycelium of the nematode-trapping fungus *Arthrobotrys oligospora* (Hyphomycetales) to cow pats." *J. of Helminthol.* 63: 115-126.

GRØNVOLD, J. & HOGH-SCHMIDT, K. (1989) "Factors influencing rain splash dispersal of infective larvae of *Ostertagia ostertagi* (Trichostrongylidae) from cow pats to the surroundings" *Vet. Parasitol.* 31(1): 57-70.

GRØNVOLD, J.; KORSHOLM, H.; WOLSTRUP, J.; NANSEN, P.; HENRIKSEN, S.A. (1985) "Laboratory Experiments to Evaluate the Ability of *Arthrobotrys oligospora* to Destroy Infective Larvae of Cooperia sp. and to Investigate the Effect of Physical Factors on the Growth of the Fungus." *J. Helminthology* 59(2): 119-125.

GRØNVOLD, J.; NANSEN, P.; HENRIKSEN, S.A.; THYLIN, J. & WOLSTRUP, J. (1988) "The capacity of the predacious fungus *Arthrobotrys oligospora* (Hyphomycetales) to reduce numbers of infective larvae of *Ostertagia ostertagi* (Trichostrongylidae) in cow pats and herbage during the grazing season in Denmark" *J. Helminthol.* 62(4): 271-280.

GRØNVOLD, J.; WOLSTRUP, J.; HENRIKSEN, S.A.; NANSEN, P. (1987) "Field Experiments on the Ability of *Arthrobotrys oligospora* (Hyphomycetales) to reduce the number of larvae of *Cooperia oncophera* (Trichostrongylidae) in cow pats and on surrounding grass." *J. Helminthol.* 61: 65-71.

GRØNVOLD, J.; WOLSTRUP, J.; NANSEN, P; HENRIKSEN, S.A.; LARSEN, M; BRESCIANI, J. (1993) "Biological control of nematode parasites in cattle with nematode-trapping fungi: a survey of Danish studies" *Vet. Parasitol.* 48: 311-325.

GRUNER, L. & CABARET, J. (1985) "Current methods for estimating parasite populations: Potential and limits to control gastrointestinal and pulmonary strongyles of sheep on pasture" *Livestock Production Science* 13: 53-70.

GRUNER, L.; PELOILLE, M.; SAUVE, C.; CORTET, J. (1985) "Animal Parasitology.- Survival and nematode-trapping activity against trichostrongyles of three species of predatory fungi after ingestion by sheep." *C.R. Acad Sc. Paris* t.300, Serie III, No 14. 1985.

GULLAND, F.M.D. & FOX, M. (1992) "Epidemiology of nematode infections of Soay sheep (*Ovis aries* L.) on St. Kilda" *Parasitology* 105: 481-492.

HAARD, K. (1968) "Taxonomic studies on the genus *Arthrobotrys* Corda." *Mycologia,* 60: 1140-1159.

HANSEN, J.W.; ZAJAC, A.M.; EVERSOLE, D.E. & GERKEN, H.J. (1989) "The effect of stocking rate and parasite control on the performance of replacement beef heifers on pasture" *Vet. Parasitol.* 34(1): 103-116.

HASHMI, H.A. & CONNAN, R.M. (1989) "Biological control of ruminant Trichostrongylids by *Arthrobotrys oligospora*, a predacious fungus" *Parasitol. Today* 5(1): 28-30.

HAYES, W.A. & BLACKBURN, F. (1966) "Studies on the nutrition of *Arthrobotrys oligospora* Fres. and *A. robusta* Dudd. II. The predaceous phase" *Ann. Appl. Biol.* 58: 51-60.

HECKER, J.F. (1969) "A simple rapid method for inserting rumen cannulae in sheep" *Aust. Vet. J.* 45: 293-4.

HEINTZ, C.E. (1978) "Assessing the Predacity of Nematode-Trapping Fungi in-vitro." *Mycologia* 70(5): 1086-1100.

HOLMES, P.H. & COOP, R.L. (1994) "Workshop summary: Pathophysiology of gastrointestinal parasites" *Vet. Parasitol.* 54: 299-303.

HYDEN, S. "The Use of Reference Substances in the Measurement of Flow in the Alimentary Tract" Ch. 4 in *Digestive Physiology and Nutrition of the Ruminant* (D. Lewis Ed.) Butterworths, Lndn. 1961.

JAFFEE, B.A.; GASPARD, J.T. & FERRIS, H. (1989) "Density-dependent parasitism of the soil-borne nematode *Criconemella xenoplax* by the nematophagous fungus *Hirsutella rhossiliensis* " *Microb. Ecol.* 17(2): 193-200.

JAFFEE, B.A.; GASPARD, J.T.; FERRIS, H. & MULDOON, A.E. (1988) "Quantification of parasitism of the soil-borne nematode *Criconemella xenoplax* by the nematophagous fungus *Hirsutella rhossiliensis*" *Soil Biol. Biochem.* 20(5): 631-636.

JAFFEE, B.A. & MULDOON, A.E. (1989) "Suppression of cyst nematode by natural infestation of a nematophagous fungus" *J. Nematol.* 21(4): 505-510.

JAFFEE, B.A.; MULDOON, A.E. & TEDFORD, E.C. (1992) "Trap Production by Nematophagous Fungi Growing from Parasitised Nematodes" *Phytopathology* 82(6): 615-620.

JAFFEE, B.; PHILLIPS, R.; MULDOON, A. & MANGEL, M. (1992) "Density-dependent host-pathogen dynamics in soil microcosms" *Ecology* 73(2): 495-506.

JANSSON, H-B. (1982) "Predacity by Nematophagous Fungi and its Relationship to the Attraction of Nematodes." *Microb. Ecol.* 8(3): 233-240.

JAROWAJA, N. (1970) "Rodzaj *Arthrobotrys* Corda. (The genus *Arthrobotrys* Corda.)" *Acta Mycologica*, 6: 337-406.

JATALA, P. (1986) "Biological Control of Plant-Parasitic Nematodes" *Ann. Rev. Phytopathol.* 24: 453-89.

JEONG, M.J. & KIM, H.K. (1988) "Isolation of nematophagous fungi against root-knot nematode and their growth in vitro" *Korean J. Appl. Entomol.* 27(3): 149-158.

KAPUR, S.; BELFIELD, W. & GIBSON, N.H.S. (1981) "The effects of fungicides on soil fungi with special reference to nematophagous species" *Pedobiologia* 21: 172-181.

KERRY, B. (1980) "Biocontrol: Fungal Parasites of Female Cyst Nematodes." *J. of Nematol.* 12(4): 253-259.

KIERAN, P.J. "Moxidectin against ivermectin-resistant nematodes - a global view" *Aust Vet J.* 71(1): 18-20.

KIM, H.K.; JEONG, J.M.; CHOO, H.Y. & PARK, C.S. (1988) "Decrease of nematode population by introduction of nematophagous fungi into the soil as affected by inoculum concentration and temperature in vitro" *Korean J. Appl. Entomol.* 27(3): 159-164.

LARSEN, M. *"Studies on the Capability of Microfungi to Destroy Animal Parasitic Nematodes"* Ph.D. Thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. 1991.

LARSEN, M. (1991) "Ability of the fungus *Pleurotus pulmonarius* to immobilise preparasitic nematode larvae" *Res. in Vet. Sci.* 51: 246-249.

LARSEN,M.; FAEDO,M. & WALLER, P.J. (1994) "The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: survey for the presence of fungi in fresh faeces of grazing livestock in Australia" *Vet. Parasitol.* 53: 275-281.

LARSEN, M. & NANSEN, P. (1990) "Effects of Oyster Mushroom *Pleurotus pulmonarius* on Preparasitic Larvae of Bovine Trichostrongyles" *Acta Vet. Scand.* 31: 509-510.

LARSEN, M.; WOLSTRUP, J.; HENRIKSEN, S.A.; DACKMAN, C.; GRØNVOLD, J. & NANSEN, P. (1991) "*In vitro* stress selection of nematophagous fungi for biocontrol of parasitic nematodes in ruminants" *J. of Helminthol.* 65: 193-200.

LARSEN, M.; WOLSTRUP, J.; HENRIKSEN, S.A.; GRØNVOLD, J. & NANSEN, P. (1992) "*In vivo* passage through calves of nematophagous fungi selected for biocontrol of parasitic nematodes" *J. of Helminthol.* 66: 137-141.

LEATHWICK, D.M.; BARLOW, N.D. & VLASSOFF, A. (1992) "A model for nematodiasis in New Zealand lambs" *Int. J. for Parasitol.* 22(6): 789-799.

LE JAMBRE, LF (1993) "Ivermectin-resistant *Haemonchus contortus* in Australia" *Aust Vet J.* 70(9): 357.

LEWIS, J.A.; LUMSDEN, D.; PAPAVIZAS, G.; HOLLENBECK, M.D.; WALTER, J.F. (1991) "Fungal formulation for biocontrol of soil-borne plant pathogens." *United States Patent* 1991. US 5, 068, 105.

LEWIS, J.A.; PAPAVIZAS, G.C. & LUMSDEN, R.D. (1991) "A new formulation system for the application of biocontrol fungi to soil" *Biocontrol Science and Technology* : 59-69.

LIGHTOWERS, M.W. (1994) "Vaccination against animal parasites" *Vet. Parasitol.* 54: 177-204.

LINFORD, M.B. & YAP, F. (1938) "Root-knot injury restricted by a nematode-trapping fungus." *Phytopathology* 28: 14-15 (Abstr.)

LINFORD, M.B. & YAP, F. (1939) "Root-knot injury restricted by a nematode-trapping fungus." *Phytopathology* 29: 596-609.

LINFORD, M.B.; YAP, F. & OLIVEIRA, J.M. (1938) "Reduction of soil populations of the root-knot nematode during decomposition of organic matter." *Soil Sci.* 45: 127-141.

LOHMANN; U, & SIKORA, R.A. (1989) "Mass production of the endoparasitic fungi *Drechmeria coniospora, Verticillium balanoides* and *Harposporium anguillulae* in liquid culture." *Nematologica* 35: 97-104.

LOVE, S.C.J.; JOHNS, W.H. & COVERDALE, O.R. (1992) "Anthelmintic resistance in sheep nematodes in the New England region of New South Wales" *Aust. Vet. J.* 69(8): 196-197.

LYSEK, G. & NORDBRING-HERTZ, B. (1981) "An endogenous rhythm of trap formation in the nematophagous fungus *Arthrobotrys oligospora*" *Planta* 152: 50-53.

MAGGENTI, A. *General Nematology* Springer-Verlag, N.Y., Heidelberg, Berlin. 1981.

MAHON, R.J. & WARDHAUGH, K.G. (1991) "Impact of dung from ivermectin-treated sheep on oogenisis and survival of adult *Lucilia cuprina*" *Aust. Vet. J.* 68(5): 173-177.

MANI, A. & ANANDAM, R.J. (1989) "Evaluation of plant leaves, oil cakes and agro-industrial wastes as substances for mass-production of the nematophagous fungus, *Paecilomyces lilacinus*" *J. Biol. Control* 3(1): 56-58.

MANI, A.; MURTHY, I.R.; KAMESWARA-RAO, P. & DEVA PRASAD, V. (1989) "Growth of *Paecilomyces lilacinus* on natural substrates and its efficacy against citrus nematode, *Tylenchulus semipenetrans*" *J. Biol. Control* 3(1): 59-61.

MANKAU, R. (1962) "Soil Fungistasis and Nematophagous Fungi." *Phytopathology* 52: 611-615.

MANKAU, R. (1980) "Biocontrol: Fungi as Nematode Control Agents." *J. of Nematol.* 12(4): 244-252.

McDOUGALL, E.I. (1948) "Studies on Ruminant Saliva I. The composition and output of sheep's saliva" *Biochem. J.* 43: 99-123.

McDOUGALL, S. (1992) "Controlled-release anthelmintic treatment of milking goats" *Aust. Vet. J.* 69(3): 64-65.

MILLER, R.E. (1972) "Protein cross-linked with phospho-nitrilic halide" U.S. Patent 3,674,509.

MILLER, R.E. (1972) "Protein cross-linked with polymerized unsaturated carboxylic acid" U.S. Patent 3,685,998.

MILLER, R.E. (1972) "Protein-triazine ruminant feed material" U.S. Patent 3,697,284.

MILLER, R.E. (1973) "Protein-sulfonyl halide complex ruminant feed material" U.S. Patent 3,711,289.

MILLER, R.E. (1973) "Protein-acrolein acetyl complex ruminant feed material" U.S. Patent 3,711,290.

MOSS, R.A. & VLASSOFF, A. (1993) "Effect of herbage species on gastro-intestinal roundworm populations and their distribution" *New Zealand J. of Agric. Res.* 36: 371-375.

MUNN, E.A.; SMITH, T.S.; GRAHAM, M.; GREENWOOD, C.A.; TAVERNOR, A.S. & COETZEE, G. (1993) "Vaccination of merino lambs against haemonchosis with membrane-associated proteins from the adult parasite" *Parasitology* 106: 63-66.

MURRAY, D.S. & WHARTON, D.A. (1990) "Capture and penetration processes of the free-living juveniles of *Trichostrongylus colubriformis* (Nematoda) by the nematophagous fungus, *Arthrobotrys oligospora" Parasitology* 101: 93-100.

NANSEN, P.; GRØNVOLD, J.; HENRIKSEN, Sv. Aa.; WOLSTRUP, J. (1986) "Predacious Activity of the Nematode-Destroying Fungus, *Arthrrobotrys oligospora,* on Preparasitic Larvae of *Cooperia oncophora* and on Soil Nematodes." *Proc. Helminthol. Soc. Wash.* 53(2): 237-243.

NANSEN, P.; GRØNVOLD, J.; HENRIKSEN, Sv. Aa.; WOLSTRUP, J. (1988) "Interactions between the Predacious Fungus *Arthrobotrys oligospora* and Third-Stage Larvae of a Series of Animal-Parasitic Nematodes." *Vet. Parasitol.* 26: 329-337.

NORDBRING-HERTZ, B. (1977) "Nematode-induced morphogenesis in the predacious fungus *Arthrobotrys oligospora" Nematologica* 23: 443-451.

NORDBRING-HERTZ, B. (1988) "Nematophagous fungi: strategies for nematode exploitation and for survival." *Microbiol. Sci.* 5: 108-116.

NORDBRING-HERTZ, B. & BRINCK, C. (1974) "Qualitative characterisation of some peptides inducing morphogenesis in the nematode-trapping fungus *Arthrobotrys oligospora" Physiol. Plant.* 31: 59-63.

NORDBRING-HERTZ, B. & MATTIASSON, B. (1979) "Action of nematode-trapping fungus shows lectin-mediated host-microorganism interaction " *Nature, London,* 281: 477-479.

O'CALLAGHAN, M.G.; MARTIN, R.R. & McFARLAND, I.J. (1992) "A natural infection of sheep with *Ostertagia ostertagi" Aust. Vet. J.* 69(1): 19-20.

OLTHOF, T.H.A. & ESTEY, R.H. (1963) "A nematotoxin produced by the nematophagous fungus, *Arthrobotrys oligospora* Fresenius." *Nature,* 197: 514-515.

OLTHOF, T.H.A. & ESTEY, R.H. (1965) "Relation of some Environmental Factors to Growth of Several Nematophagous Hyphomycetes." *Can. J. Microbiol.* 11: 939-945.

OSMAN, A.A. & VIGLIERCHIO, D.R. (1988) "Efficacy of biologically active agents as nontraditional nematicides for *Meloidogyne javanica" Rev. Nematol.* 11(1): 93-98.

PANDEY, V.S. (1973) "Predatory Activity of Nematode-Trapping Fungi against the Larvae of *Trichostrongylus axei* and *Ostertagia ostertagi* : A Possible Method of Biological Control." *J. Helminthol.* 47(1): 35-48.

PAPAVIZAS, G.C.; DUNN, M.T.; LEWIS, J.A. & BEAGLE-RISTAINO, J. (1984) "Liquid fermentation technology for experimental production of biocontrol fungi" *Phytopathology* 74(10): 1171-1175.

PELIOLLE, M. (1979) "Etude des Hyphomycetes predateurs de Nematodes rencontres sur une prarie du Limousin: Morphologie-physiologie, frequence et distribution." Thesis, l'Université de Rennes, pp. 106.

PERRIN, D.D. & DEMPSEY, B. *Buffers for pH and Metal Ion Control* Chapman & Hall, Lond. 1974.

POPE, D.G. (1975) "Factors to be considered in the formulation of Pharmaceuticals for oral administration to ruminant animals" *Aust. J. Pharmaceutical Science* NS4(3): 65-78.

PRAMER, D. & STOLL, N.R. (1959) "Nemin: a morphogenic substance causing trap formation by predaceous fungi" *Science* 129: 966-967.

PRITCHARD, R.K. (1990) "Anthelmintic resistance in nematodes: extent, recent understanding and future directions for control and research" *Int. J. Parasitol.* 20: 515-523.

PRITCHARD, R.K. (1994) "Anthelmintic resistance" *Vet. Parasitol.* 54: 259-268.

REES, G. (1950) "Observations on the vertical migrations of the third-stage larvae of *Haemonchus contortus* (Rud.) on experimental plots of *Lolium perenne* S24, in relation to Meteorological and Micrometeorological factors" *Parasitology* 40: 127-143.

RILEY, W.A. *Introduction to the Study of Animal Parasites and Parasitism* Burgess Publishing Co. Minneapolis. 1949.

RODRIGUEZ-KABANA, R. & MORGAN-JONES, G. (1987) "Biological control of nematodes: Soil amendments and microbial antagonists" *Plant and Soil* 100: 237-247.

ROSE, C.H. & JACOBS, D.E. (1990) "Epidemiology of *Nematodirus* species infections of sheep in a subarctic climate: development and persistence of larvae on herbage" *Res. in Vet. Sci.* 48: 327-330.

ROSE, J.H. & SMALL, A.J. (1985) "The distribution of the infective larvae of sheep gastro-intestinal nematodes in soil and on herbage and the vertical migration of *Trichostrongylus vitrinus* larvae through the soil" *J. of Helminthol.* 59: 127-135.

ROSENSWEIG, W.D.; PREMACHANDRAN, D.; PRAMER, D. (1985) "Role of Trap Lectins in the Specificity of Nematode Capture by Fungi." *Can. J. Microbiol.* 31(8): 693-695.

ROUBAUD, E. & DESCAZEAUX, J. (1939) "Action des certains champignons prédateurs sur les larves de Strongylidés du cheval." *Bull. Soc. Path. exot.* 32: 290-294.

ROUBAUD, E. & DESCHIENS, R. (1939) "Destruction des larves infectieuses d'Ankylostomes et d'Anguillules intestinales par *Dactylella ellipsospora.*"

ROUBAUD, E. & DESCHIENS, R. (1941a) "Action des Hyphomycètes prédateurs sur les larves de Synthétocaules et de Bunostomes." *Bull. Soc. Path. exot.* 34: 127-130.

ROUBAUD, E. & DESCHIENS, R. (1941b) "Essais relatifs à la prophylaxie de l'anguillulose du mouton par l'usage des Hyphomycètes prédateurs du sol." *C.R. Soc. Biol. Paris* 135: 687-690.

RUMBALL, P.J. (1991) "Management effects on growth and roundworm burdens of autumn-born lambs" *New Zealand J. of Agric. Res.* 34: 201-206.

RYDER, M.H. & JONES, D.A.(1990) "Biological control of crown gall" in *Biological control of soil-borne plant pathogens* (edited by D. Hornby) :45-63. Wallingford, Oxon; UK

SAXENA, G.; DAYAL, R. & MUKERJI, K.G. (1989) "Nutritional studies on nematode-trapping fungi" *Folia Microbiol.* 34(1): 42-48.

SAXENA, G. & LYSEK, G. (1993) "Observation of nematophagous fungi in natural soils by fluorescence microscopy and their correlation with isolation" *Mycol. Res.* 97(8): 1005-1011.

SAYRE, R.M. (1980) "Biocontrol: *Bacillus penetrans* and related parasites of Nematodes." *J. of Nematol.* 12(4): 260-270.

SAYRE, R.M. & WALTER, D.E. (1991) "Factors affecting the efficacy of natural enemies of nematodes" *Ann. Rev. Phytopathol.* 29: 149-66.

SCHENK, S.; KENDRICK, W.B. & PRAMER, D. (1977) "A new nematode-trapping hyphomycete and a reevaluation of *Dactylaria* and *Arthrobotrys* " *Can. J. of Botany*, 55: 977-985.

SCHENK, S. & PRAMER, D. (1975) "The Effects of Volatile Compounds from Nematodes on Trap Formation by a Nematode-Trapping Fungus" *Applied Microbiology* 30: 496-497.

SKLAN, D. (1992) "A note on production responses of lactating ewes to calcium soaps of fatty acids" *Anim. Prod.* 55: 288-291.

SMEAL, M.G. "Bases and Approaches for Control of Nematodes in Cattle" Chapter 9 in: *Biology and Control of Endoparasites* edited by Symons, Donald and Dineen, Academic Press, 1982.

SMEAL, M.G. & HENDY, G.A. (1972) "A technique for the recovery of strongyloid infective larvae from pasture" *J. of Helminthol.* 46: 201-211.

SMITH,G. & GRENFELL, B.T. (1994) "Modelling of parasite populations: gastrointestinal nematode models" *Vet. Parasitol.* 54: 127-143.

SOPRANOV, F.F. (1958) *Predacious Hyphomycetes and their Application in the Control of Pathogenic Nematodes.* Translated from Russian, Israel Program for Scientific Translations, Jerusalem 1966. U.S. Dept. of Commerce, Springfield, Va.22151

SOUTHCOTT, W.H.; HEATH, D.D. & LANGLANDS, J.P. (1967) "Relationship of nematode infection to efficiency of wool production" *J. Brit. Grasslands Soc.* 22: 117-120.

SOUTHCOTT, W.H.; ROE, R. & NEWTON-TURNER, H. (1959) "Grazing management of native pastures in the New England region of New South Wales. II. The effect of size of flock on pasture and sheep production with special reference to internal parasites and grazing behaviour." *Aust. J. Agric. Res* 13: 880-893.

STEAR, M.J. & MURRAY, M. (1994) "Genetic resistance to parasitic disease: particularly of resistance in ruminants to gastrointestinal nematodes" *Vet. Parasitol.* 54: 161-176.

STIRLING, G.R. (1989) "Organic amendments for control of root-knot nematode (*Meloidogyne incognita*) on ginger" *Australas Plant Pathol.* 18(2): 39-44.

STIRLING, G.R. & KERRY, B.R. (1983) "Antagonists of the Cereal Cyst Nematode *Heterodera avenae* in Australian Soils." *Aust. J. Exp. Agric. Anim. Husb.* 23(122): 318-324.

STIRLING, G.R.; SHARMA, R.D.; PERRY, J. (1990) "Attachment of *Pasteuria penetrans* spores to the root-knot nematode *Meloidogyne javanica* in soil and its effects on infectivity" *Nematologica* 36: 246-252.

STIRLING, G.R. & WACHTEL, M.F. (1980) "Mass Production of *Bacillus penetrans* for the Biological Control of Root-Knot Nematodes." *Nematologica* 26: 308-312.

STIRLING, G.R. & WEST, L.M. (1991) "Fungal parasites of root-knot nematode eggs from tropical and sub-tropical regions of Australia" *Aust. Plant Pathol.* 20(4): 149-154.

STURHAN, D. (1988) "New host and geographical records of nematode-parasitic bacteria of the *Pasturia penetrans* group." *Nematologica* 34: 350-356.

SUTTLE, M. & BREBNER, J. (1990) "Associations of breed and nematodiasis with intake of a free-access mineral mixture by grazing lambs." *Proc. Nutrition Soc.* 49(3): 220A.

SUTTLE, N.F.; KNOX, D.P.; JACKSON, F.; COOP, R.L.; ANGUS, K.W. (1992) "Effects of dietary molybdenum on nematode and host during *Trichostrongylus vitrinus* infection in lambs." *Res. Vet. Science* 52(2): 224-229.

SUTTLE, N.F.; KNOX, D.P.; JACKSON, F.; COOP, R.L. (1992) "Effects of dietary molybdenum on nematode and host during *Haemonchus contortus* infection in lambs." *Res. Vet. Science* 52(2): 230-235.

SYKES, A.R. & POPPI, D.P. (1988) "Effect of concurrent infection with *Ostertagia circumcincta* and *Trichostrongylus colubriformis* on the performance of growing lambs consuming fresh herbage" *J. Agric. Sci. Camb.* 110: 531-541.

SYMONS, L.E.A. *Pathophysiology of endoparasitic infection compared with ectoparasitic infestation and microbial infection* Academic Press, Australia. 1989.

TAYLOR, R. (1993) "Overcoming worm resistance to chemicals" *Rural Research* 159: 114.

TEDFORD, E.C.; JAFFEE, B.A. & MULDOON, A.E. (1992) "Effect of Soil Moisture and Texture on Transmission of the Nematophagous Fungus *Hirsutella rhossiliensis* to Cyst and Root-Knot Nematodes" *Phytopathology* 82: 1002-1007.

THORN, R.G. & BARRON, G.L. (1984) "Carnivorous Mushrooms" *Science* 224: 76-78.

TIMPER, P. & KAYA, H.K. (1989) "Role of the Second-Stage Cuticle of Entomogenous Nematodes in Preventing Infection by Nematophagous Fungi" *J. of Invertebrate Pathology* 54: 314-321.

TRIBE, H.T. (1980) "Prospects for the Biological Control of Plant Parasitic Nematodes." *Parasitol.* 81(3): 619-639.

TUNLID, A.; JOHANSSON, T. & NORDBRING-HERTZ, B. (1991) "Surface polymers of the nematode-trapping fungus *Arthrobotrys oligospora*" *J. of General Microbiology* 137: 1231-1240.

VAN ADRICHEM, P.W.M. & VOGT, J.E. (1993) "The effect of isolation and separation on the metabolism of sheep" *Livest. Prod. Sci.* 33: 151-159.

VAN DEN BOOGERT, P.H.J.F.; DIJKSTERHUIS, J.; VELVIS, H. & VEENHUIS, M. (1992) "Adhesive knob formation by conidia of the nematophagous fungus *Drechmeria coniospora*" *Antonie van Leeuwenhoek* 61: 221-229.

VAN OORSCHOT, C.A.N. (1985) "Taxonomy of the *Dactylaria* complex, V. A review of *Arthrobotrys* and allied genera." *Studies in Mycology*, 26: 61-96.

VAN SLOUN, P.; NICOLAY, R.; LOHMANN, U. & SIKORA, R.A. (1990) "Anfalligkeit von entomopathogenen Nematoden gegenuber nematodenfangenden und endoparasitaren Pilzen" *J. Phytopathology* 129: 217-227.

VAN SOEST, P.J. *Nutritional Ecology of the Ruminant* O & B Books, Oregon, USA. 1982.

VEENHUIS, M.; HARDER, W. & NORDBRING-HERTZ, B. (1989) "Occurrence and metabolic significance of microbodies in trophic hyphae of the nematophagous fungus *Arthrobotrys oligospora.*" *Antonie van Leeuwenhoek* 56: 241-249.

VEENHUIS, M.; SJOLLEMA, K.; NORDBRING-HERTZ, B. & HARDER, W. (1989) "An improved method for light- and electron microscopial studies of nematode/fungal interactions." *Antonie van Leeuwenhoek* 55: 361-368.

VEENHUIS, M.; VAN WIJK, C.; WYSS, U.; NORDBRING-HERTZ, B. & HARDER, W. (1989) "Significance of electron dense microbodies in trap cells of the nematophagous fungus *Arthrobotrys oligospora*" *Antonie van Leeuwenhoek* 56: 251-261.

WALL, R. & STRONG, L. (1987) "Environmental consequences of treating cattle with the drug ivermectin" *Nature* 327: 418-421.

WALLER, P.J. (1992) "Towards sustainable nematode parasite control of livestock" *Vet. Parasitol.*

WALLER, P.J. (1992) "Prospects for biological control of nematode parasites of ruminants" *New Zealand Vet. J.* 40: 1-3.

WALLER, P.J.; DOBSON, R.J. & AXELSEN, A. (1988) "Anthelmintic resistance in the field: Changes in resistance status of parasitic populations in response to anthelmintic treatment" *Aust. Vet. J.* 65(12): 376-379.

WALLER, P.J. & FAEDO, M. (1992) "The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Screening Studies." *Vet. Parasitol.* 49: 285-297.

WALLER, P.J. & LARSEN, M. (1993) "The role of nematophagous fungi in the biological control of nematode parasites of livestock" *Int. J. Parasitol.* 23(4): 539-546.

WALLER, P.J.; LARSEN, M.; FAEDO, M. & HENNESSEY, D.R. (1994) "The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: in vitro and in vivo studies" *Vet. Parasitol.* 51: 289-299.

WHARTON, D.A. & MURRAY, D.S. (1990) "Carbohydrate/lectin interactions between the nematophagous fungus, *Arthrobotrys oligospora,* and the infective juveniles of *Trichostrongylus colubriformis* (Nematoda)" *Parasitology* 101: 101-106.

WHITLOCK, H.V. (1948) *J. Counc. Sci. Ind. Res. Aust.* 21: 177-180.

WHITLOCK, J.H.; CROFTON, H.D. & GEORGI, J.R. (1972) "Characteristics of parasite populations in endemic trichostrongylidosis" *Parasitology* 64: 413-427.

WORONIN, M. (1870) *Sphaeria lemneae, Sordaria coprophila, arthrobotrys oligospora.* In Bettr, Morph. Physiol. Pilze. III. (A. de Bary and M. Woronin, Eds.) Abhandl. Senckenbergische naturf. Ges. 7: 325-360.

WYBORN, C.H.E.; PRIEST, D.; DUDDINGTON, C.L. (1969) "Selective Technique for the Determination of Nematophagous Fungi in Soils." *Soil Biol. Biochem.* 1(1): 101-102.

YOUNG, R.R. (1983) "Populations of free-living stages of *Ostertagia ostertagi* and *O. circumcincta* in a winter rainfall region" *Aust. J. Agric. Res.* 34: 569-81.

YOUNG, R.R. & TRAJSTMAN, A.C. (1980) "A rapid technique for the recovery of strongyloid infective larvae from pasture and soil samples" *Parasitology* 80: 425-431.

ZOPF, W. (1888) "Zur kenntnis der Infections-Krankheiten nieder Tier und Pflanzen." *Nova Acta Leop-Carol.* 52: 314-376.

APPENDICES

1. SURVEY OF SHEEP GRAZING PROPERTIES

Properties included in survey:

1. Mona Vale, Ross

Sites sampled:

Paddock 1. Chapmans Marsh

Description: 500Ha, native and improved pasture, sub clover. Open stony hills and river terraces, basalt rock, light red-brown silty duplex soil, darker in lower patches, mostly unimproved pasture.

1. Near gate. Flats and hillside.
2. Southern boundary. Gentle southerly slopes, a few trees.
3. South-east corner, open, native grass, sub clover.
4. Eastern end, tussocks and black soil.
5. North-east near river, variable soil.
6. Northern river flats, silty clay loam.
7. Pastured river terraces. Light red-brown soil.

Paddock 2. River flats

Description: 240Ha, Improved pastures on variable soil, some as above, some heavy, black, puggy, Flood irrigated.

1. Moist loamy soil.
2. Irrigated area, variable soil.
3. Black puggy soil, hard angular, blocky peds.
4. Dark soil along river bank.

2. Charleton, Ross

Sites sampled:

Paddock 1. Anstey's Hill Run

Description: 240Ha, bush run, hilly, light tree cover, some open grassland, soils variable from red-brown silty loam to bleached sandy loam, some heavy clay loam, no sub clover, mostly native grass.

1. Sandy soil with tussocks, northern boundary.
2. Red-brown soil, hillside.
3. Pale grey soil under light bush, north-east boundary.
4. West side, open grassland, grey soil.
5. River flats, north-eastern end, darker silty loam.

6. Wickham's boundary, good grassy slope, grey loam.
7. Stony open hilltop, light grey silt loam.
8. South slope, grey silty loam.

Paddock 2. Cattle Paddock

Description: Improved pasture, variable soil, all open grassland, flat.

1. Eastern end, stony (vesicular basalt) variable soil.
2. Variable grey-brown soil, blocky peds.
3. Dark grey loamy soil.
4. Brown loamy soil under lush pasture.
5. Black puggy soil with gilgais.
6. Black heavier loam.

3. Vaucluse, Conara

Sites sampled:

Paddock 1. Ram Paddock

1. North end, red soil under ryegrass/white clover.
2. North end, dark soil.
3. Transect 1.
4. Transect 2.

Paddock 2. Bullock paddock.

1. Transect north-south.
2. South end
3. Salty patch
4. East side beside salt pan.

4. Glasslough, Epping Forest.

Sites sampled:

Paddock 1. Roadside paddock.

1. Transect.

Paddock 2. Middle Creek Paddock.

1. West end
2. East end

Paddock 3. Marshes

1. Transect.
2. Swampy ground under reeds
3. Banks above wet ground
4. Corbie-infested patch.
5. Sandy soil banks where sheep camp.

6. Sandy soil under pine tree near gate.

5. Leverington, Epping Forest.
Sites sampled:

Paddock 1. Strawberry paddock.

1. Sandy side, covered in pasture.
2. Black soil side, eaten out by corbies and cockchafers.

Paddock 2. River flats.

1. Transect 1. Western half.
2. Transect 2. Eastern half.
3. Sandy bank beside river.

6. Viewpoint, Isis.
Sites sampled:

Paddock 1. Slip paddock.

1. Transect 1.
2. Transect 2.

Paddock 2. Middle paddock (considered wormy by farmer)

1. Black soil.
2. Transect.

7. Cullenswood, St Marys
Sites sampled:

1. River flats and banks, improved pasture.
2. Plain behind river flats, black soil.
3. Plain behind river flats, brown sandy soil.
4. Foothills, toeslopes.
5. High ground beside Break-O-Day River.
6. Recently cultivated paddock, rape crop.

8. Malahide, Fingal.
Sites sampled:

1. Esk flood plain beside Mathinna Road.
2. Esk flood plain river flats.
3. "Clean" paddock 1., high side of Mathinna Rd.
4. "Clean" paddock 2., grazed by ewes, lambs and cattle.
5. Giles Bridge, under barley crop.
6. Giles Bridge, under peppermint crop.
7. Green Hills, top and slope.

8. Green Hills, black soil at foot of slope.
9. Break-O-Day to Esk Highway transect.
10. Paddock south side of Esk Highway (3 Sisters)

9. Rostrevor, Avoca.
Sites sampled:
1. Below house towards river.
2. Under trees in forest behind house.
3. Gravelly soil under oat crop.
4. Finer soil under oats.
5. South of Highway, fine grey soil.
6. South of highway, coarser soil.

10. Scamander, Home paddock, J. Bushing.

11. Chain of Lagoons, Stoneyford creek, black soil.

12. Lilla Villa, East Coast.
Sites sampled:
1. Transect across flat paddock of grey soil.
2. Red (lateritic) soil on hill.
3. Gravelly soil, newly cleared ground.
4. Yards beside shearing shed.
5. River flats next to shearing shed.
6. Roadside clearing, Lilla Villa bridge.

13. Greenlawn, Bicheno.
Sites sampled:
1. Entrance paddock, grey soil.
2. Black soil on flats.
3. Paddock beside driveway, grey soil.

14. Springvale, Cranbrook.
Sites sampled:
1. Chocolate loam under poppies.
2. Chocolate loam under lucerne.
3. Red-brown soil under pasture.
4. Iron-stone soil under pasture.
5. Chocolate loam under Italian rye and clover.

15. Dolphin Sands, sand under tussocks by roadside.

16. Belmont, paddock beside Dolphin Sands Road.

17. Swansea Beach, picnic spot below golf course.

18. Kelvedon, Swansea.

Sites sampled:

1. Transect across paddock at Swansea end.
2. Transect across paddock on black soil.
3. Grey soil under oats and rape.
4. Very old paddock around ruined house.
5. Paddock in front of ruined house.
6. Run country west of cleared paddocks.

		RESULTS OF GLASSHOUSE TRIAL 19/11/92					
	Counts at First Harvest (100s)						
	Inoculum in:	Spray & Soil	Soil only	Spray only	Control		
	Block 1	168	340	80	440		
		34	240	128	148		
		110	232	90	110		
	Block 2	20	220	68	66		
		26	250	100	500		
		120	142	18	240		
	Block 3	136	100	44	92		
		32	166	160	438		
		20	90	72	208		
	Block 4	260	210	88	164		
		180	106	64	145		
		160	54	18	218		
	Block 5	142	152	58	126		
		110	236	40	144		
		60	50	36	240		
	Anova Two-Factor With Replication						
	Summary						
		Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*	
	Block 1						
	Count	3.00	3 00	3 00	3.00	12.00	
	Sum	312.00	812 00	298 00	698.00	2120 00	
	Average	104.00	270 67	99 33	232 67	706 67	
	Variance	4516.00	3621 33	641 33	32601 33	41380 00	
	Block 2						
	Count	3 00	3 00	3 00	3 00	12.00	
	Sum	166.00	612.00	186 00	806.00	1770.00	
	Average	55.33	204 00	62 00	268 67	590.00	
	Variance	3145.33	3108.00	1708 00	47705 33	55666 67	
	Block 3						
	Count	3 00	3.00	3 00	3 00	12 00	
	Sum	188 00	356 00	276 00	738 00	1558.00	
	Average	62 67	118.67	92 00	246 00	519 33	
	Variance	4069.33	1705.33	3664 00	31012.00	40450 67	
	Block 4						
	Count	3.00	3 00	3.00	3.00	12 00	
	Sum	600 00	370.00	170.00	527.00	1667.00	
	Average	200 00	123.33	56 67	175 67	555.67	
	Variance	2800.00	6309 33	1265 33	1434.33	11809 00	
	Block 5						
	Count	3.00	3 00	3.00	3.00	12.00	
	Sum	312.00	438 00	134.00	510 00	1394 00	
	Average	104.00	146 00	44.67	170 00	464 67	
	Variance	1708 00	8676.00	137 33	3756 00	14277.33	
	Total						
	Count	15 00	15 00	15 00	15 00		
	Sum	1578 00	2588 00	1064.00	3279.00		
	Average	526.00	862 67	354.67	1093.00		
	Variance	16238 67	23420 00	7416.00	116609 00		
	ANOVA						
Source of Variation		*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Sample	24681.07	4.00	6170.27	0.75438664	0 56108616	2.60597233
	Column	198066.32	3.00	66022.11	8.07196793	0.00025337	2.83874613
	Interaction	94768.27	12.00	7897.36	0 96554329	0 49606692	2.00346051
	Within	327167.33	40.00	8179.18			
	Total	644682.98	59.00				

RESULTS OF GLASSHOUSE TRIAL 26/11/92

Counts at Second Harvest (100s)

Inoculum in:	Spray & Soil	Soil only	Spray only	Control
Block 1	168	200	88	255
	53	143	90	282
	150	154	113	153
Block 2	6	376	103	180
	31	505	152	230
	56	386	6	294
Block 3	62	238	36	219
	74	125	84	340
	31	263	112	512
Block 4	22	335	58	212
	12	64	16	264
	23	118	31	500
Block 5	112	153	144	391
	89	430	127	339
	36	205	105	355

Anova Two-Factor With Replication

Summary

	Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*
Block 1					
Count	3	3	3	3	12
Sum	371	497	291	690	1849
Average	123 666667	165.666667	97	230	616.333333
Variance	3826 33333	914 333333	193	4629	9562.66667
Block 2					
Count	3	3	3	3	12
Sum	93	1267	261	704	2325
Average	31	422 333333	87	234.666667	775
Variance	625	5150 33333	5521	3265.33333	14561.6667
Block 3					
Count	3	3	3	3	12
Sum	167	626	212	1071	2076
Average	55 6666667	208 666667	70 6666667	357	692
Variance	492.333333	5406 33333	1477 33333	21679	29055
Block 4					
Count	3	3	3	3	12
Sum	57	517	105	976	1655
Average	19	172.333333	35	325 333333	551 666667
Variance	37	20574 3333	453	23557 3333	44621.6667
Block 5					
Count	3	3	3	3	12
Sum	237	788	376	1085	2486
Average	79	262 666667	125 333333	361.666667	828 666667
Variance	1519	21676.3333	382 333333	709 333333	24287
Total					
Count	15	15	15	15	
Sum	925	3695	1245	4526	
Average	308 333333	1231.66667	415	1508.66667	
Variance	8499.66667	53721.6667	8026 66667	53840	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	38237 2333	4	9559 30833	1.56597017	0.20206252	2 60597233
Column	636675.383	3	212225.128	34.7659275	3.1653E-11	2.83874613
Interaction	180442 367	12	15036 8639	2 46328286	0 01631281	2.00346051
Within	244176	40	6104.4			
Total	1099530 98	59				

RESULTS OF GLASSHOUSE TRIAL 4/12/92

Counts at Third Harvest (100s)

Inoculum in.	Spray & Soil	Soil only	Spray only	Control
Block 1	88	82	16	141
	19	68	12	152
	4	67	10	305
Block 2	8	295	109	282
	21	121	74	69
	18	121	9	331
Block 3	17	72	16	690
	42	34	22	110
	9	10	27	123
Block 4	20	313	17	228
	20	52	17	245
	34	44	19	181
Block 5	42	87	14	103
	43	144	45	55
	48	237	54	112

Anova Two-Factor With Replication

Summary

	Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*
Block 1					
Count	3.00	3 00	3.00	3.00	12 00
Sum	111 00	217 00	38.00	598.00	964.00
Average	37.00	72 33	12 67	199.33	321.33
Variance	2007.00	70 33	9.33	8404.33	10491.00
Block 2					
Count	3.00	3.00	3 00	3 00	12 00
Sum	47 00	537.00	192 00	682 00	1458.00
Average	15 67	179.00	64 00	227 33	486 00
Variance	46 33	10092.00	2575 00	19402 33	32115 67
Block 3					
Count	3.00	3 00	3.00	3.00	12 00
Sum	68.00	116.00	65 00	923 00	1172 00
Average	22.67	38 67	21 67	307.67	390.67
Variance	296 33	977 33	30 33	109676 33	110980 33
Block 4					
Count	3 00	3.00	3.00	3 00	12 00
Sum	74 00	409.00	53.00	654 00	1190 00
Average	24.67	136 33	17 67	218.00	396 67
Variance	65 33	23424 33	1.33	1099.00	24590.00
Block 5					
Count	3.00	3.00	3.00	3 00	12 00
Sum	133.00	468.00	113 00	270.00	984 00
Average	44 33	156 00	37.67	90 00	328.00
Variance	10 33	5733 00	440.33	939 00	7122 67
Total					
Count	15 00	15 00	15.00	15.00	
Sum	433 00	1747.00	461 00	3127.00	
Average	144 33	582 33	153.67	1042 33	
Variance	2425 33	40297.00	3056.33	139521.00	

ANOVA

Source of Vanation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	13252.93	4.00	3313 23	0 35760813	0.83720433	2.60597233
Column	327512.80	3.00	109170.93	11.7831764	1 1415E-05	2 83874613
Interaction	108377.87	12.00	9031.49	0 97479818	0.48802843	2 00346051
Within	370599.33	40.00	9264.98			
Total	819742.93	59.00				

		RESULTS OF GLASSHOUSE TRIAL 11/12/92					
	Counts at Fourth Harvest (100s)						
	Inoculum in:	Spray & Soil	Soil only	Spray only	Control		
	Block 1	12	7	2	39		
		4	25	4	8		
		25	31	4	40		
	Block 2	4	50	18	32		
		6	58	14	120		
		21	100	1	22		
	Block 3	12	17	1	3		
		15	121	10	19		
		6	13	6	15		
	Block 4	13	26	3	2		
		13	14	1	50		
		3	13	1	25		
	Block 5	5	9	4	35		
		9	80	13	39		
		6	18	4	24		
	Anova: Two-Factor With Replication						
	Summary						
		Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*	
	Block 1						
	Count	3.00	3 00	3 00	3 00	12.00	
	Sum	41.00	63 00	10 00	87.00	201.00	
	Average	13 67	21 00	3 33	29.00	67.00	
	Variance	112 33	156 00	1 33	331 00	600.67	
	Block 2						
	Count	3 00	3 00	3 00	3 00	12.00	
	Sum	31 00	208 00	33 00	174 00	446 00	
	Average	10 33	69 33	11.00	58 00	148.67	
	Variance	86 33	721.33	79.00	2908 00	3794.67	
	Block 3						
	Count	3 00	3.00	3 00	3.00	12 00	
	Sum	33.00	151.00	17 00	37.00	238.00	
	Average	11.00	50.33	5.67	12 33	79 33	
	Variance	21 00	3749.33	20.33	69.33	3860.00	
	Block 4						
	Count	3 00	3 00	3 00	3 00	12 00	
	Sum	29.00	53 00	5.00	77 00	164 00	
	Average	9 67	17.67	1 67	25 67	54.67	
	Variance	33.33	52.33	1 33	578 33	663.33	
	Block 5						
	Count	3 00	3 00	3 00	3 00	12 00	
	Sum	20 00	107 00	21.00	98.00	246 00	
	Average	6 67	35.67	7.00	32.67	82 00	
	Variance	4.33	1494 33	27.00	60 33	1586.00	
	Total						
	Count	15 00	15.00	15.00	15 00		
	Sum	154.00	582.00	86 00	473 00		
	Average	51 33	194.00	28 67	157.67		
	Variance	257 33	6173.33	129.00	3945 00		
	ANOVA						
Source of Variation		*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Sample	3997 33	4 00	999.33	1 90	0.12884565	2 61
	Column	11620.58	3.00	3873.53	7.37	0.00047902	2.84
	Interaction	5083 33	12 00	423 61	0 81	0 64198117	2.00
	Within	21009 33	40.00	525.23			
	Total	41710.58	59 00				

RESULTS OF GLASSHOUSE TRIAL 17/12/92

Counts at Fifth Harvest (100s)

Inoculum in	Spray & Soil	Soil only	Spray only	Control
Block 1	4	11	4	23
	1	18	1	41
	5	17	2	22
Block 2	5	22	5	22
	14	39	2	45
	7	7	2	11
Block 3	9	27	10	20
	5	19	9	23
	1	5	8	19
Block 4	9	24	4	21
	23	4	3	43
	64	9	1	31
Block 5	5	4	3	22
	4	8	5	6
	6	32	4	15

Anova Two-Factor With Replication

Summary

	Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*
Block 1					
Count	3 00	3 00	3 00	3.00	12.00
Sum	10 00	46 00	7.00	86 00	149 00
Average	3 33	15 33	2 33	28.67	49 67
Variance	4.33	14 33	2 33	114.33	135 33
Block 2					
Count	3.00	3 00	3 00	3 00	12 00
Sum	26.00	68 00	9 00	78 00	181 00
Average	8 67	22.67	3 00	26 00	60.33
Variance	22 33	256 33	3.00	301.00	582 67
Block 3					
Count	3 00	3 00	3 00	3 00	12 00
Sum	15 00	51 00	27.00	62 00	155 00
Average	5 00	17 00	9.00	20 67	51 67
Variance	16 00	124 00	1.00	4.33	145.33
Block 4					
Count	3.00	3 00	3 00	3 00	12 00
Sum	96.00	37.00	8 00	95 00	236 00
Average	32 00	12 33	2 67	31.67	78.67
Variance	817 00	108 33	2 33	121 33	1049.00
Block 5					
Count	3 00	3.00	3.00	3 00	12 00
Sum	15.00	44.00	12.00	43.00	114 00
Average	5 00	14.67	4 00	14 33	38 00
Variance	1 00	229.33	1.00	64.33	295.67
Total					
Count	15 00	15.00	15 00	15.00	
Sum	162.00	246.00	63 00	384.00	
Average	54.00	82 00	21 00	121 33	
Variance	860.67	732 33	9 67	805.33	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	686.17	4 00	171 54	1.55381944	0 20534321	2.60597233
Column	3261.25	3.00	1087.08	9.84676932	5.4317E-05	2.83874613
Interaction	1883.17	12.00	156.93	1.42147242	0.1965814	2.00346051
Within	4416.00	40.00	110.40			
Total	10246.58	59.00				

		RESULTS OF GLASSHOUSE TRIAL					
	TOTAL OF ALL WEEKLY COUNTS (100s)						
	Inoculum in:	Spray & Soil	Soil only	Spray only	Control		
	Block 1	440	640	190	898		
		111	494	235	631		
		294	501	219	630		
	Block 2	43	963	303	582		
		98	973	342	964		
		222	756	36	898		
	Block 3	236	454	107	1024		
		168	465	265	930		
		67	381	225	877		
	Block 4	324	908	170	627		
		248	240	101	747		
		284	238	70	955		
	Block 5	306	405	223	677		
		255	898	230	583		
		156	542	203	746		
	TOTALS	3252	8858	2919	11769		
	MEANS	216 8	590 53	194.6	784.6		
	Anova: Two-Factor With Replication						
	Summary						
		Spray & Soil	*Soil only*	*Spray only*	*Control*	*Total*	
	Block 1						
	Count	3.00	3 00	3.00	3.00	12.00	
	Sum	845.00	1635 00	644.00	2159 00	5283.00	
	Average	281.67	545 00	214 67	719 67	1761 00	
	Variance	27174 33	6781.00	520 33	23852 33	58328.00	
	Block 2						
	Count	3 00	3.00	3 00	3.00	12.00	
	Sum	363 00	2692 00	681 00	2444.00	6180 00	
	Average	121 00	897.33	227 00	814.67	2060 00	
	Variance	8407 00	15006 33	27741 00	41689.33	92843 67	
	Block 3						
	Count	3.00	3 00	3 00	3 00	12.00	
	Sum	471 00	1300 00	597 00	2831 00	5199.00	
	Average	157.00	433 33	199.00	943 67	1733 00	
	Variance	7231 00	2084 33	6748.00	5542 33	21605.67	
	Block 4						
	Count	3.00	3.00	3 00	3.00	12.00	
	Sum	856 00	1386.00	341 00	2329 00	4912 00	
	Average	285 33	462.00	113.67	776.33	1637 33	
	Variance	1445.33	149188 00	2620 33	27541.33	180795 00	
	Block 5						
	Count	3.00	3 00	3 00	3.00	12.00	
	Sum	717 00	1845 00	658 00	2006.00	5224 00	
	Average	239 00	615.00	218.67	668 67	1741.33	
	Variance	5817 00	64759 00	196.33	6694 33	77466 67	
	Total						
	Count	15 00	15.00	15.00	15 00		
	Sum	3252 00	8858 00	2919 00	11769 00		
	Average	1084 00	2952.67	973.00	3923.00		
	Variance	50074.67	237818.67	37826.00	105319 67		
	ANOVA						
	Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
	Sample	76954.10	4.00	19238 52	0.8926582	0.47726912	2.60597233
	Column	3769092 60	3.00	1256364.20	58.294688	1.1634E-14	2.83874613
	Interaction	561189.23	12.00	46765.77	2.16990896	0.03335649	2.00346051
	Within	862078.00	40.00	21551 95			
	Total	5269313.93	59 00				

RESULTS AND ANALYSIS OF DATA FROM FIRST SPRAY TRIAL

Total Nemas Recovered from Plots (100s)

Spores/ml					
0	13	40	31	36	10
15	28	12	20	27	51
150	37	26	29	13	29
750	43	27	63	26	26
1500	15	17	13	23	6

Anova: Single-Factor

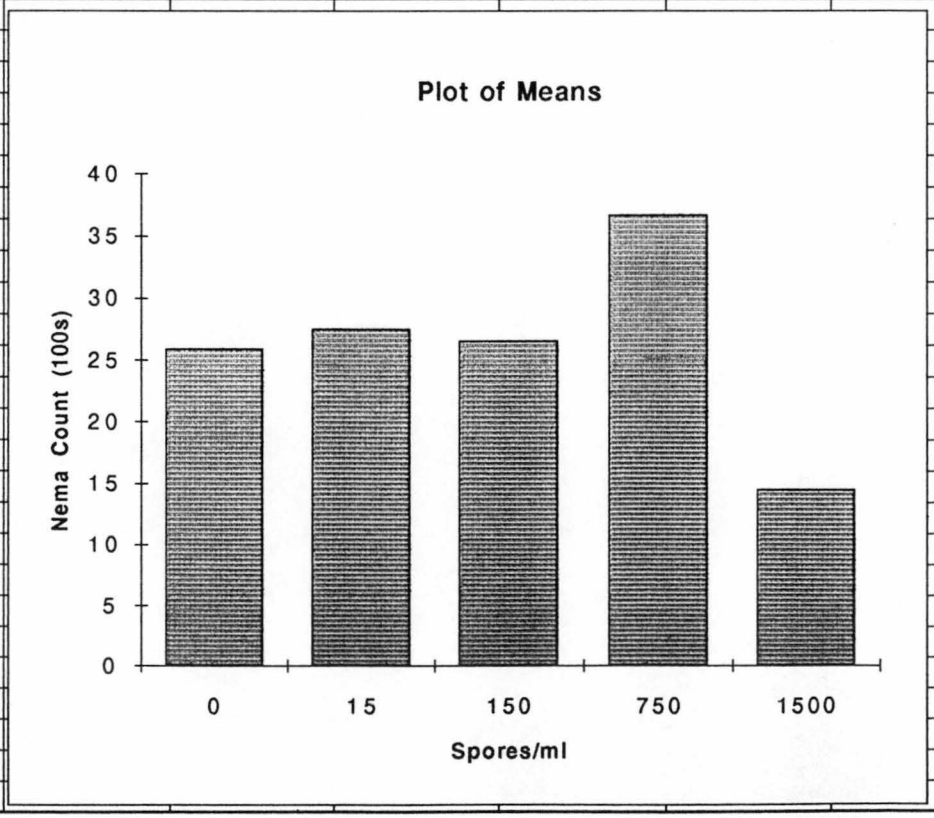
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
0	5	130	26	183
15	5	138	28	207
150	5	133	27	76
750	5	184	37	267
1500	5	73	15	40

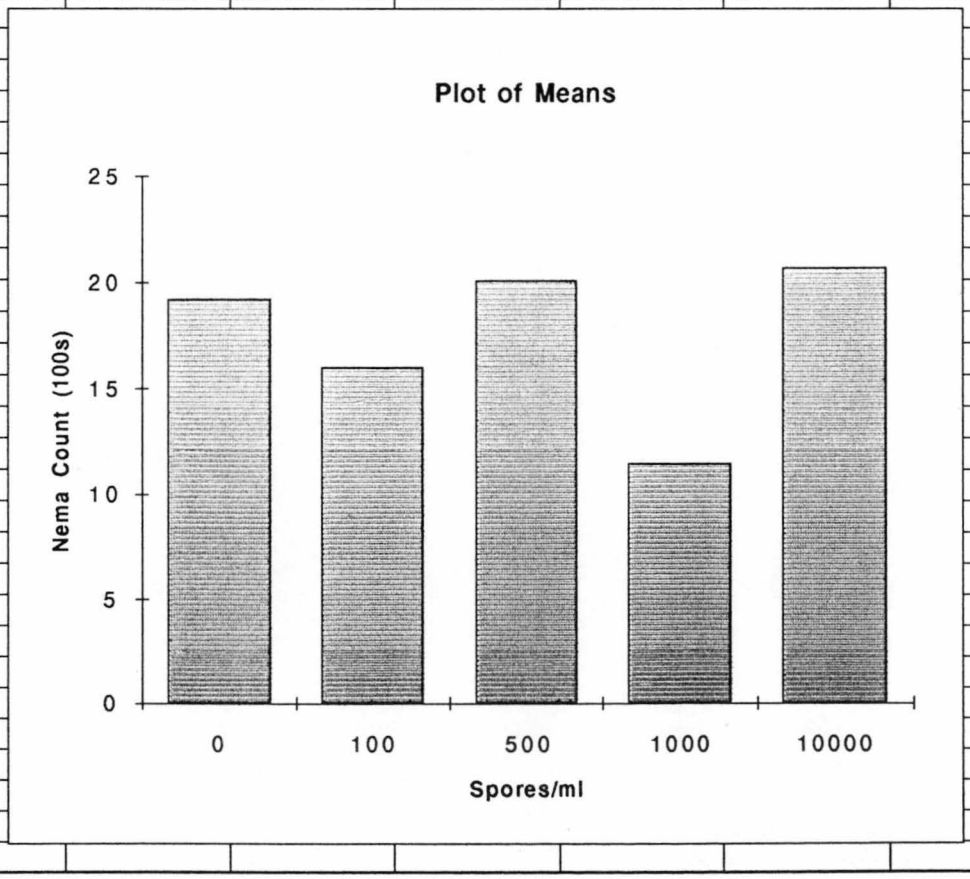
ANOVA

Source of Variation

	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Group	1245	4	311	2	0.13128662	2.86608071
Within Groups	3093	20	155			
Total	4338.06	24				



	RESULTS AND ANALYSIS OF DATA FROM SECOND SPRAY TRIAL					
	Total Nemas Recovered from Plots (100s)					
Spores/ml	0	100	500	1000	10000	
	8	10	10	17	6	
	16	26	36	12	12	
	42	9	9	20	32	
	10	8	8	6	43	
	22	28	39	4	12	
Anova: Single-Factor						
Summary						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0	5	96	19.2	183.95		
100	5	80	16	101.625		
500	5	100.5	20.1	247.175		
1000	5	57.5	11.5	45.375		
10000	5	103.5	20.7	247.075		
ANOVA						
Source of Variation						
	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Group	290.7	4	72.675	0.44034779	0.77798397	2.86608071
Within Groups	3300.8	20	165.04			
Total	3591.5	24				



RESULTS AND ANALYSIS OF DATA FROM THIRD SPRAY TRIAL

Total Nemas Recovered per Plot (100s)

Spores/ml	0	100	500	1000	10000
	49	28	109	120	16
	150	109	37	222	63
	86	72	50	107	52
	169	77	114	152	59
	138	176	83	154	52

Anova: Single-Factor

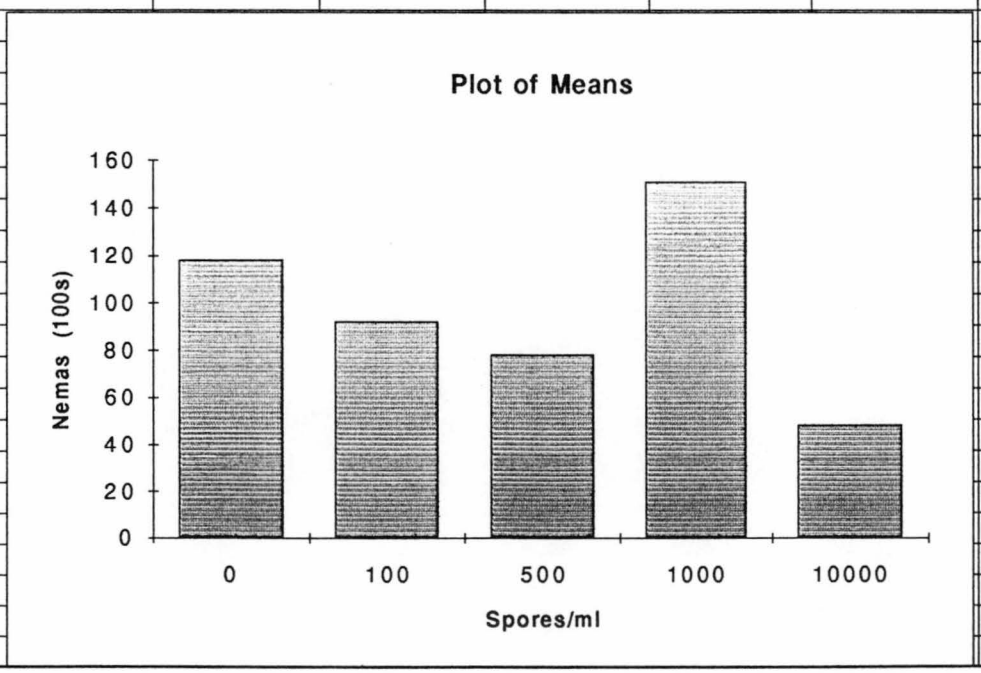
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
0	5	591	118	2431.825
100	5	461	92	3033.425
500	5	391	78	1195.2
1000	5	755	151	1986.3
10000	5	242	48	350.3

ANOVA

Source of Variation

	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Group	30462.46	4	7615.615	4.23228447	0.01212191	2.86608071
Within Groups	35988.2	20	1799.41			
Total	66450.66	24				



RESULTS AND ANALYSIS OF DATA FROM FOURTH SPRAY TRIAL

log Dose	0	3	4	5	6
	228	102	45	33	65
	285	104	106	95	65
	316	258	207	103	71
	346	308	346	186	126
	378	499	410	267	148

Anova: Single-Factor

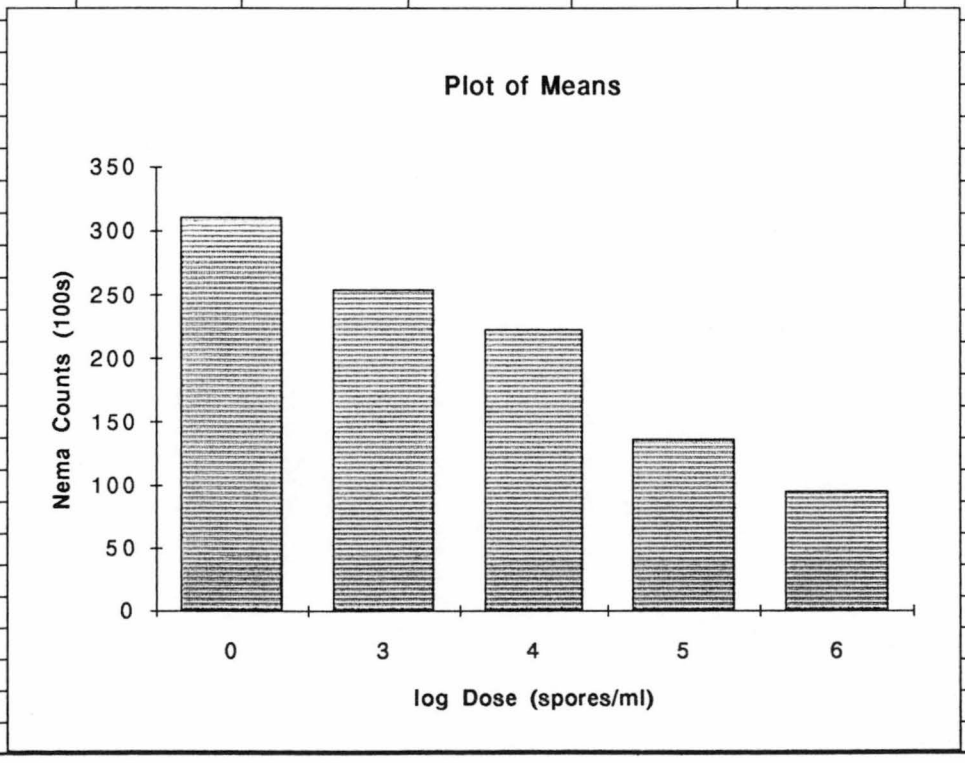
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
0	5	1553	310.6	3325.8
3	5	1271	254.2	27140.2
4	5	1114	222.8	23931.7
5	5	684	136.8	8259.2
6	5	475	95	1536.5

ANOVA

Source of Variation

	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Group	153169.04	4	38292.26	2.98256986	0.04402677	2.86608071
Within Groups	256773.6	20	12838.68			
Total	409942.64	24				



RESULTS AND ANALYSIS OF DATA FROM TIMING TRIAL

Number of worm larvae in plots (100s)

Faecal Age (d	14	7	3	0	-3	-7
	300	413	415	65	267	331
	309	191	119	71	122	281
	181	421	300	148	105	203
	416	354	631	126	102	251
	256	463	199	65	488	50

Anova: Single-Factor

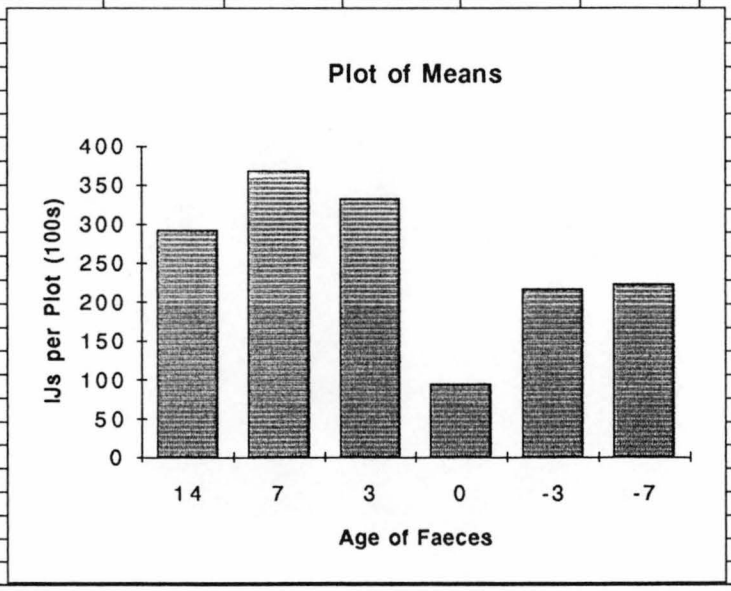
Summary

Groups	*Count*	*Sum*	*Average*	*Variance*
14	5	1462	292.4	7336.3
7	5	1842	368.4	11345.8
3	5	1664	332.8	40092.2
0	5	475	95	1536.5
-3	5	1084	216.8	27683.7
-7	5	1116	223.2	11535.2

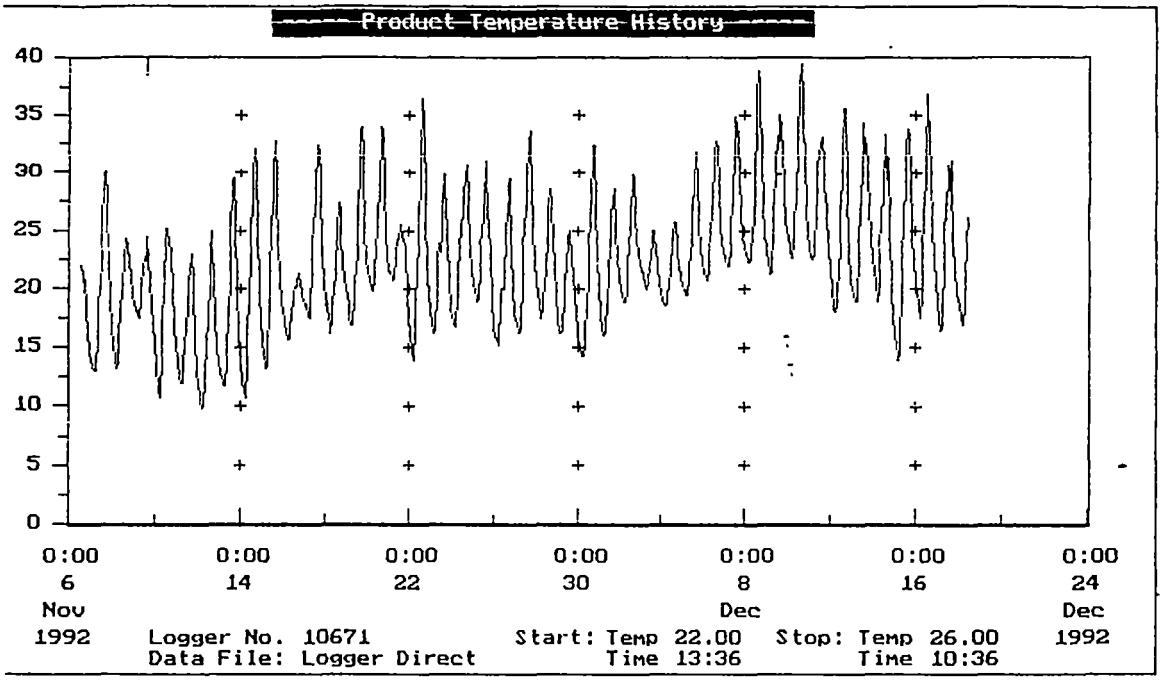
ANOVA

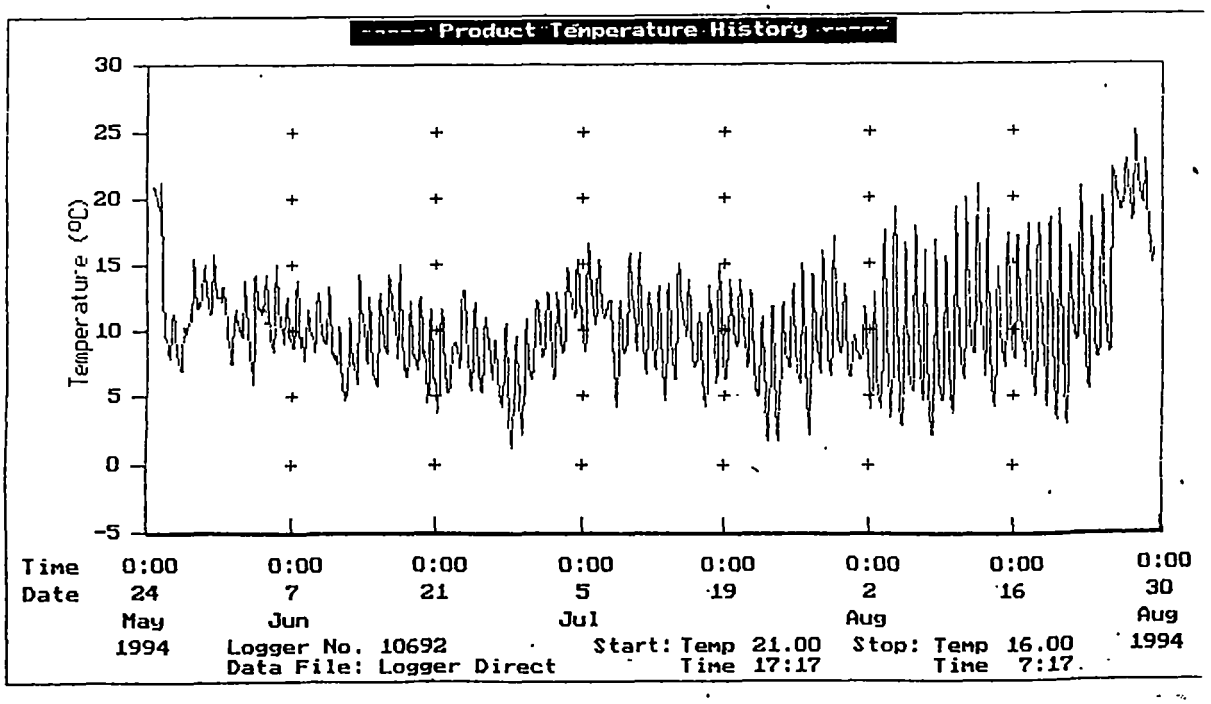
Source of Variation

	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between Gro	241906.567	5	48381.3133	2.91659555	0.0339489	2.62065214
Within Grou	398118.8	24	16588.2833			
Total	640025.367	29				



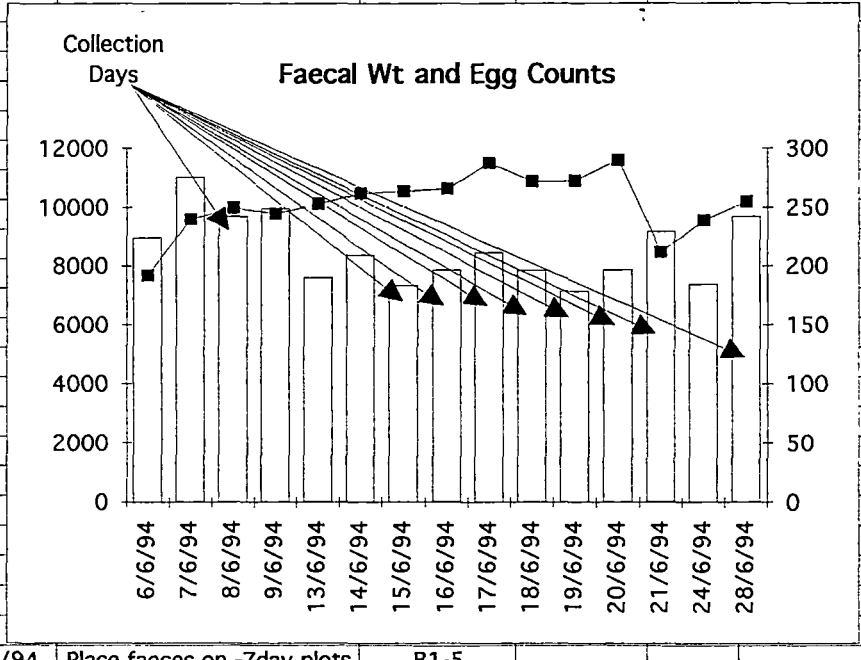
Temperatures in pasture tubs - during Inoculum Trial, top, and final Concentration Rate and Timing Trials, below. Printout is from a Delphi data logger.





spray trials calendar

DATE	PROCEDURE	COMMENTS	Mean EPG	Std Dev.	Faeces Wt (g)
6/6/94	Sample Sheep	single sample	8970		192
7/6/94	Sample sheep	Five samples	11024	1202	240
8/6/94	Sample sheep	Five samples	9714	1308	250
9/6/94	Sample sheep	Five samples	9966	1105	245
13/6/94	Sample sheep	Five samples	7602	1098	253
14/6/94	Sample sheep	Five samples	8370	918	262
15/6/94	Sample sheep	Five samples	7338	2172	264
16/6/94	Sample sheep	Five samples	7866	1941	266
17/6/94	Sample sheep	Five samples	8454	790	288
18/6/94	Sample sheep	Five samples	7860	1222	272
19/6/94	Sample sheep	Five samples	7140	1388	272
20/6/94	Sample sheep	Five samples	7872	1661	290
21/6/94	Sample sheep	Five samples	9180	363	212
24/6/94	Sample sheep	Five samples	7362	1367	239
28/6/94	Sample sheep	Five samples	9696	822	255



14/6/94	Place faeces on -7day plots	B1-5			
18/6/94	Place faeces on -3day plots	C1-5			
20/6/94	Spore suspension made	>300M per ml			
21/6/94	Place faeces on 0day plots	D1-5			
21/6/94	Set up Spray Trial				
24/6/94	Place faeces on +3day plots	E1-5			
28/6/94	Place faeces on +7day plots	F1-5			
2/8/94	Harvest Plots	(D;G-J)1-5			
3/8/94	Count Infectives				
4/8/94	Harvest Plots	(A-F)1-5			
5/8/94	Count Infectives				